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PRINCIPAL INVESTIGATOR: Ernesto R.Bongarzone

CONTRACTING ORGANIZATION:
University of Illinois, Chicago, IL. 60612-4305

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# REPORT DOCUMENTATION PAGE

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Ernesto R. Bongarzone

E-Mail: ebongarz@uic.edu

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**14. ABSTRACT**

Relapsing remitting multiple sclerosis (RRMS) is demyelinating disease that affects both men and women and is characterized by cycles of acute myelin loss, followed by remission with active myelin repair. The mechanism and the cellular source for remyelination are still in discussion, but there is evidence for the involvement of adult oligodendrocyte progenitor cells(OPCs). In this study, we hypothesized that circulating precursor cells identified by the presence of the cell surface marker CD133 may be of relevance during MS and for CNS repair processes. We measured the abundance of CD133+ and CD34+ cells in peripheral blood collected from RRMS patients and healthy controls. Our results showed that circulating CD34+ cells were not significantly affected by the disease. In contrast, CD133+ cells were significantly reduced in the RRMS patients recruited in this study. Interestingly, when CD133+ values from RRMS women were compared to RRMS men, we found that women had significantly lower values than the men ($p<0.029$). The fraction of hematopoietic CD133+ cells that were positive for the CD34+ marker was significantly elevated in RRMS patients.

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multiple sclerosis, neuroinflammation, stem cells, neurogenesis, hematopoiesis, T-cells.

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## INTRODUCTION & BODY.

Multiple Sclerosis (MS) is a widespread neurological disorder (Rosati, 2001; Compston and Coles, 2008; Trojano et al., 2012; Evans et al., 2013) affecting more women than men (~3 to 1) (Compston and Coles, 2008). To date, MS is without a defined etiology and is considered an autoimmune disorder affecting the central nervous system (CNS), adopting various degrees of severity from relapsing remitting (RRMS) to progressive forms.

Demyelinated lesions or "plaques" are found randomly distributed in the MS CNS (Frohman et al., 2006) and evidence of spontaneous remyelination has been presented (Keirstead and Blakemore, 1999; Barnett and Prineas, 2004; Prineas and Parratt, 2012). However, the cellular and molecular mechanisms regulating remyelination are still largely unclear. Various sources of remyelinating cells may contribute to repair. For example, adult quiescent oligodendrocyte progenitor cells and other neural precursor cells (e.g. derived from the subventricular zone) appear to be recruited for myelin repair (Patrikios et al., 2006; Nait-Oumesmar et al., 2007; Nait-Oumesmar et al., 2008). In addition, infiltration of bloodborne cells in the MS brain underlines the possibility that circulating undifferentiated precursors may also contribute to neural repair (Porat et al., 2006), similarly to bloodborne monocytes colonizing the brain and becoming *bona fide* microglia (Soulet and Rivest, 2008).

Past experiments found that peripheral blood mononuclear cells (PBMCs) isolated from a small group of patients with RRMS expressed the Olig2 gene –a transcription factor involved in the generation of oligodendrocytes-. This finding suggested that mobilization of NPs with oligodendrocyte characteristics may occur in some MS patients. We speculated that NPs can be mobilized from two sources: 1) during disease, ruptures or transient openings of the BBB permit NPs to exit the nervous system and be circulated by the blood. These NPs are born or derive from endogenous NSCs residing within the nervous system; 2) during disease, the damaged nervous system signals or beacons the recruitment of peripheral stem cells to adopt a neural phenotype and thus be mobilized into the blood. In both scenarios, our application assumed that blood-borne neural precursors (BNPs) had the ability to either neuroprotect the MS nervous system and/or engage in remyelination by differentiating in myelinating oligodendrocytes once infiltrated in the nervous system of the MS patient. The goal of this application was to determine the identity of mobilized BNPs and to characterize their potential to engage in remyelination in a mouse model.

Our work focalized on blood proliferative primitive progenitor cells (pPC) characterized by the co-expression of cell surface markers CD34 and CD133 (Krause et al., 1996; Miraglia et al., 1997). CD133, a 120-kDa glycosylated pentaspan integral surface protein (Miraglia et al., 1997; Mizrak et al., 2008), identifies a population of undifferentiated precursor cells present in various tissues, including blood, fetal liver, kidney, prostate and CNS (Yin et al., 1997; Lee et al., 2005; Corti et al., 2007; Mizrak et al., 2008). Circulating CD133+CD34+ pPC are pluripotential being capable to generate

hematopoietic lineages and also neural lineages (Uchida et al., 2000; Schwartz et al., 2003; Jang et al., 2004; Hombach-Klonisch et al., 2008; Kuci et al., 2008; Hafizi et al., 2013). Because of this plasticity, progenitor cells expressing CD133 are of interest for their potential role in neural repair. To what extent CD133+ cells are affected in MS is unclear.

Blood samples were collected as proposed in our SOW and used for analysis of the expression of neural genes (SOW task #1, 2 and 3), the presence of progenitor cells with neural potential (SOW task #2), and the in vivo and in vitro behavior (SOW task #2, 3)

**Methodology employed.**

**Ethics statement.** The study was approved by the Office for the Protection of Research Subjects of the Institutional Review Board at University of Illinois at Chicago (# 2001-0721), and by the Research and Clinical Trials Administration Office of the Institutional Review Board at Rush University Medical Center (# 09092107-IRB01). Informed written consent was signed by each subject.

**Patients and clinical data.** For this study, we prospectively enrolled RRMS patients diagnosed according to the revised McDonald's criteria (McDonald et al., 2001; Polman et al., 2011). Age matched healthy volunteers were selected from students, University personnel, and colleagues of the laboratory and the hospital. These controls were probed to exclude a diagnosis of MS or other neurological diseases. Final blood samples were collected from 20 RRMS patients (15 women and 5 men; mean ± standard deviation age of 42 ± 9 years) and 23 healthy volunteers (11 women and 12 men; 38 ± 12 years) were used for flow cytometry. Four patients were in the midst of a relapse at the time of blood draw while others were in remission for 2 months to 5 years. Blood samples from 8 additional RRMS patients (2 women and 6 men; 43 ± 9 years) and from 4 controls (2 women and 2 men; 38 ± 10 years) were used for *in vitro* experiments. These additional patients were all in remission for 1 to 3 years. Table II summarizes the demographic and clinical information of all MS patients used in this study.

**Isolation of peripheral blood mononuclear cells.** Blood was drawn by venipuncture between 9 am and 2 pm (before lunch) by qualified personnel at the MS clinic of Rush University and the Neurology clinic of University of Illinois at Chicago. PBMCs were isolated from 15 to 30 ml blood collected in EDTA tubes (BD, Franklin Lakes, NJ) using the Ficoll-Paque protocol. Briefly, blood was diluted 1:1 in sterile 2 mM EDTA PBS to decrease viscosity. A gradient of Ficoll-Paque (GE Healthcare Biosciences, Pittsburgh, PA) was prepared by centrifugation with a swinging bucket centrifuge model 5810 (Eppendorf, Hamburg, Germany) at 1,000 g for 10 min, using an acceleration value of 9, in frit-containing tubes (Greiner Bio-One, Kremsmuenster, Austria). Diluted blood was loaded onto the gradient and spun at 1,000 g for 15 min without applying brake. PBMCs were carefully recovered and washed twice in 10 ml of 2 mM EDTA, PBS by spinning first at 300 g, then at 200 g to eliminate Ficoll-Paque and platelets. Cells were counted in a Neubauer chamber (Reichert, Buffalo, NY) and adjusted to 1 million cells per ml in 0.5% BSA, 2 mM EDTA, PBS.

**Flow cytometry.** PBMCs were pelleted at 3,000 g for 5 min and resuspended in 100 µl of human FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Each tube containing one million PBMCs was incubated for 15-30 min at 4°C with 1 µg of either a mouse anti-human CD133 antibody-PE (phycoerythrin), a mouse anti-human CD34 antibody-FITC (fluorescein isothiocyanate), or both. Negative controls included incubations with IgG1-PE and IgG2a-FITC. All antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). After two washes at 300 g, cells were transferred to fluocytometry tubes and incubated with 7-Amino-Actinomycin D (BD, Franklin Lakes, NJ) to label dead cells, which were subtracted from the total cell count. About 200,000 events were counted in G2 using a LSR I flow cytometer (Beckton Dickinson, Franklin Lakes, NJ). Counted cells were expressed as a percentage of the total gated lymphocytic fraction of PBMCs. Raw data analysis was performed with Summit Version 4.3 software (Dako, Glostrup, Denmark). The fraction of double labeled CD133+CD34+ cells from total CD133+ cells was calculated by first subtracting the appropriate controls (IgG-PE and IgG-FITC) from the corresponding fluorescent CD133 and CD34 values. The corrected values were used in the percentage formula: [(CD133+CD34+)/(CD133+)] X 100.

**Magnetic bead sorting.** CD133+ cells were purified from PBMCs using a CD133 MicroBead Kit for human cells and Magnetic-Activated Cell Sorting (MACS®) separation columns (both from Miltenyi Biotec), following the manufacturer's recommendations. Typically, 100,000 to 200,000 cells were isolated per subject for immediate use in the assays described below.

**Colony Forming Unit Assay.** The colony forming unit assay was performed on CD133+ cells purified by MACS from 3 RRMS patients and 3 healthy volunteers, immediately mixed in medium and plated in three independent experiments in triplicate. Cells were cultured in human Methocult® medium H4434 Classic containing the recombinant human growth factors erythropoietin, stem cell factor, interleukin-3 and granulocyte macrophage colony stimulating factor, all from StemCell Technologies (Vancouver, Canada). Briefly, about 10,000 CD133+ cells per patient or control were resuspended in 300 µl **of** Iscove's Modified Dulbecco's Medium supplemented with 2% fetal bovine serum. Six ml of Methocult medium was slowly added to the cell suspension, which was plated using 16 gauge blunt-end needles in triplicates of 35 mm dishes (3,000 cells per 2 ml). Cultures were incubated at 37°C under 5% $CO_2$, and left undisturbed per the manufacturer instructions. After a 15-day culture, the frequency and type of colonies from each of the 3 independent experiments (3 MS and 3 controls per experiment) performed in triplicate were scored and grouped according to their morphological features.

**Cell Proliferation Assay.** Cell proliferation assay was performed by plating 180,000 MACS isolated CD133+ cells from a subject in a six-well plate coated with a layer of Poly-L-lysine (10 µg/ml) underneath a top layer of laminin (10 µg/ml) at a density of 30,000 cells per well. Cells were first plated in Minimum Essential **Medium** (MEM), and allowed to attach overnight to the well bottom in a 37°C, 5% $CO_2$ incubator. One half of

the plate (3 wells) was then supplemented with growth factors to promote cell proliferation: 150 ng/ml of Fms-like tyrosine kinase-3 ligand (FLT3-Ligand; R&D Systems, Minneapolis, MN), 150 ng/ml of stem cell factor, and 20 ng/ml of interleukin-3 (Cell Signaling, Danvers, MA) in CellGro® serum-free stem cell growth medium (CellGenix, Freiburg, Germany). The other half of the plate (3 wells) was incubated in CellGro® medium and omitting the growth factors as control reference. Cells were cultured for 6 days before being photographed, measured for viability, and counted using the ImageJ software subroutine. Six independent experiments (3 MS patients and 3 healthy subjects) were carried in triplicate.

**Cell Migration Assay.** Cell migration assay was performed using a 96-well format Neuroprobe ChemoTX® Disposable Chemotaxis System with a filter pore size of 8 μm (Receptor Technologies, Adderbury, U.K.). Briefly, 180,000 CD133+ cells in PBS, MACS purified from 3 MS patients and 3 healthy volunteers, were incubated with a 10 μM solution of calcein AM pre-dissolved in DMSO (Molecular Probes, Life Technologies, Grand Island, NY). After 15 min incubation, cells were washed twice in PBS by centrifugation at 300 g at 4°C for 5 min, and resuspended in MEM at a concentration of 30,000 cells per 50 μl. Then 29 μl of plain MEM medium or MEM plus 10 ng/ml stromal cell-derived factor-1$\alpha$ (SDF-1$\alpha$, R&D Systems) was added to the wells. The 8 μm pore filter was placed on top of the 96-well plate and 50 μl of the calcein-labeled cell suspension was placed above the filter membrane according to the manufacturer's specifications. The 96-well plate andfilter unit was covered with the provided lid and incubated at 37°C for 2h under 5% $CO_2$. After two hours the fluorescent signal of the cells at the bottom of wells were measured at 515 nm in a plate reader. Six independent experiments (3 MS patients and 3 healthy subjects) were carried in triplicate.

**Gene expression analysis.** RNA was isolated from mononuclear cells from 16 female MS patients and from 23 healthy female volunteers. Total RNA was isolated using the RNAqueous-Micro Kit (Ambion). Twenty micrograms of fluorescence-labeled amplified RNA were prepared using the Amino Allyl MessageAmp II Kit (Ambion). We used 2 μg of each sample to hybridize -in triplicate- with full genome Human OneArray (Phalanx Biotech) according to the manufacturer's recommendations. The arrays were scanned using ScanArray Lite (Perkin Elmer). Scanned images were analyzed with ScanArray System software to obtain gene expression ratios. Statistical analysis was performed using LIMMA (Smyth,G.K. 2004. *Statistical Applications in Genetics and Molecular Biology* 3, Article 3). To gain more accuracy and statistical from our full analysis, we compared labeled RNA from individual female or male patients to labeled RNA from pooled either female or male controls (reference RNA). We analyzed our data using the RRC facility, as above, which included scanning, normalization and statistical analysis of the data.

**Statistics**. All values are expressed as mean±standard deviation of percentages. All statistical analyses were performed using non-parametric tests: Mann-Whitney U Test, Spearman correlation and Kruskal-Wallis analysis of variance (GraphPad Prism).

## RESULTS

### Gene expression analysis of neural genes in MS blood (UNDER SOW# 1, 2 &3)

We obtained a set of signals representing putative genes that are significantly de-regulated in all the male patients or in all the female patients. For the females, a total of 58 putative genes were significantly de-regulated. Out of these, 16 putative genes showed on average lower expression than in control females, while 42 putative genes showed higher expression than in control females. For the males, a total of 127 putative genes showed significant de-regulation. Out of these, 54 putative genes showed on average lower expression than in control males, while 73 putative genes showed higher expression than in control males.

Our microarray data produced significant difference between females and the corresponding female pool control as a group. A list of deregulated genes is presented in table I below, and summarized in a volcano plot in figure 1 below.

Table I. List of deregulated genes in MS female patients

| Gene_symbol | Gene_description |
|---|---|
| COL28A1 | Collagen alpha-1(XXVIII) chain Precursor [Source:UniProtKB/Swiss-Prot;Acc:Q2UY09] |
| XXbac-B135H6.15 | processed transcript |
| ZC3H12B | Probable ribonuclease ZC3H12B (EC 3.1.-.-)(Zinc finger CCCH domain-containing protein 12B)(MCP-induced protein 2) [Source:UniProtKB/Swiss-Prot;Acc:Q5HYM0] |
| GNGT2 | Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-T2 Precursor (G gamma-C)(G-gamma-8)(G-gamma-9) [Source:UniProtKB/Swiss-Prot;Acc:O14610] |
| C1orf220 | Putative uncharacterized protein C1orf220 [Source:UniProtKB/Swiss-Prot;Acc:Q5T0J3] |
| CDK2AP2 | Cyclin-dependent kinase 2-associated protein 2 (CDK2-associated protein 2)(DOC-1-related protein)(DOC-1R) [Source:UniProtKB/Swiss-Prot;Acc:O75956] |
| C2orf18 | Transmembrane protein C2orf18 Precursor [Source:UniProtKB/Swiss-Prot;Acc:Q8N357] |
| BDNF | Brain-derived neurotrophic factor Precursor (BDNF)(Abrineurin) [Source:UniProtKB/Swiss-Prot;Acc:P23560] |
| NOTCH4, | Notch 4 |
| HCN4 | Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 [Source:UniProtKB/Swiss-Prot;Acc:Q9Y3Q4] |
| ATP8B3 | Probable phospholipid-transporting ATPase IK (EC 3.6.3.1)(ATPase class I type 8B member 3) [Source:UniProtKB/Swiss-Prot;Acc:O60423] |
| CALML3 | Calmodulin-like protein 3 (Calmodulin-related protein NB-1)(CaM-like protein)(CLP) [Source:UniProtKB/Swiss-Prot;Acc:P27482] |
| ARL10 | ADP-ribosylation factor-like protein 10 [Source:UniProtKB/Swiss-Prot;Acc:Q8N8L6] |
| TSP50 | Testis-specific protease-like protein 50 Precursor (Cancer/testis antigen 20)(CT20) [Source:UniProtKB/Swiss-Prot;Acc:Q9UI38] |
| F2RL2 | Proteinase-activated receptor 3 Precursor (PAR-3)(Thrombin receptor-like 2)(Coagulation factor II receptor-like 2) [Source:UniProtKB/Swiss-Prot;Acc:O00254] |
| FAM47DP,PRRG1 | NA |
| ALAS2 | 5-aminolevulinate synthase, erythroid-specific, mitochondrial Precursor (EC 2.3.1.37)(5-aminolevulinic acid synthase)(Delta-aminolevulinate synthase)(Delta-ALA synthetase)(ALAS-E) [Source:UniProtKB/Swiss-Prot;Acc:P22557] |
| TMEM45B | Transmembrane protein 45B [Source:UniProtKB/Swiss-Prot;Acc:Q96B21] |
| SLC25A37 | Mitoferrin-1 (Mitochondrial iron transporter 1)(Solute carrier family 25 member 37)(Mitochondrial solute carrier protein) [Source:UniProtKB/Swiss-Prot;Acc:Q9NYZ2] |
| TMEM159 | Promethin (Transmembrane protein 159) [Source:UniProtKB/Swiss-Prot;Acc:Q96B96] |
| AIP | AH receptor-interacting protein (AIP)(Aryl-hydrocarbon receptor-interacting protein)(Immunophilin |

| | |
|---|---|
| | homolog ARA9)(HBV X-associated protein 2)(XAP-2) [Source:UniProtKB/Swiss-Prot;Acc:O00170] |
| MVP | Major vault protein (MVP)(Lung resistance-related protein) [Source:UniProtKB/Swiss-Prot;Acc:Q14764] |
| MTHFS | 5-formyltetrahydrofolate cyclo-ligase (EC 6.3.3.2)(5,10-methenyl-tetrahydrofolate synthetase)(Methenyl-THF synthetase)(MTHFS) [Source:UniProtKB/Swiss-Prot;Acc:P49914] |
| GPR172A | Porcine endogenous retrovirus A receptor 1 Precursor (PERV-A receptor 1)(Protein GPR172A) [Source:UniProtKB/Swiss-Prot;Acc:Q9HAB3] |
| RNASEH2C | Ribonuclease H2 subunit C (RNase H2 subunit C)(Ribonuclease HI subunit C)(Aicardi-Goutieres syndrome 3 protein)(AGS3)(RNase H1 small subunit) [Source:UniProtKB/Swiss-Prot;Acc:Q8TDP1] |
| HSD17B10 | 3-hydroxyacyl-CoA dehydrogenase type-2 (EC 1.1.1.35)(3-hydroxyacyl-CoA dehydrogenase type II)(Type II HADH)(3-hydroxy-2-methylbutyryl-CoA dehydrogenase)(EC 1.1.1.178)(17-beta-hydroxysteroid dehydrogenase 10)(Mitochondrial ribonuclease P protein 2)(Mitochondrial RNase P protein 2)(Endoplasmic reticulum-associated amyloid beta-peptide-binding protein)(Short-chain type dehydrogenase/reductase XH98G2) [Source:UniProtKB/Swiss-Prot;Acc:Q99714] |
| GATAD2A | Transcriptional repressor p66-alpha (Hp66alpha)(GATA zinc finger domain-containing protein 2A) [Source:UniProtKB/Swiss-Prot;Acc:Q86YP4] |
| PIP5K2A | Phosphatidylinositol-5-phosphate 4-kinase type-2 alpha (EC 2.7.1.149)(Phosphatidylinositol-5-phosphate 4-kinase type II alpha)(1-phosphatidylinositol-5-phosphate 4-kinase 2-alpha)(PtdIns(5)P-4-kinase isoform 2-alpha)(PIP4KII-alpha)(Diphosphoinositide kinase 2-alpha)(PtdIns(4)P-5-kinase B isoform)(PIP5KIII)(PtdIns(4)P-5-kinase C isoform) [Source:UniProtKB/Swiss-Prot;Acc:P48426] |
| NFAT5 | Nuclear factor of activated T-cells 5 (NF-AT5)(T-cell transcription factor NFAT5)(Tonicity-responsive enhancer-binding protein)(TonE-binding protein)(TonEBP) [Source:UniProtKB/Swiss-Prot;Acc:O94916] |
| RAB37 | Ras-related protein Rab-37 [Source:UniProtKB/Swiss-Prot;Acc:Q96AX2] |
| DHX15 | Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 (EC 3.6.1.-)(DEAH box protein 15)(ATP-dependent RNA helicase #46) [Source:UniProtKB/Swiss-Prot;Acc:O43143] |
| TTC38 | Tetratricopeptide repeat protein 38 (TPR repeat protein 38) [Source:UniProtKB/Swiss-Prot;Acc:Q5R3I4] |
| SMEK1 | Serine/threonine-protein phosphatase 4 regulatory subunit 3A (SMEK homolog 1) [Source:UniProtKB/Swiss-Prot;Acc:Q6IN85] |
| SH2D1B | SH2 domain-containing protein 1B (EWS/FLI1-activated transcript 2)(EAT-2) [Source:UniProtKB/Swiss-Prot;Acc:O14796] |
| PGDS | Prostaglandin-H2 D-isomerase Precursor (EC 5.3.99.2)(Lipocalin-type prostaglandin-D synthase)(Glutathione-independent PGD synthetase)(Prostaglandin-D2 synthase)(PGD2 synthase)(PGDS2)(PGDS)(Beta-trace protein)(Cerebrin-28) [Source:UniProtKB/Swiss-Prot;Acc:P41222] |
| TBX21 | T-box transcription factor TBX21 (T-box protein 21)(Transcription factor TBLYM)(T-cell-specific T-box transcription factor T-bet) [Source:UniProtKB/Swiss-Prot;Acc:Q9UL17] |
| FCRL6 | Fc receptor-like protein 6 Precursor (FcR-like protein 6)(FcRL6)(Fc receptor homolog 6)(FcRH6)(IFGP6) [Source:UniProtKB/Swiss-Prot;Acc:Q6DN72] |
| CCNT1 | Cyclin-T1 (CycT1)(Cyclin-T) [Source:UniProtKB/Swiss-Prot;Acc:O60563] |
| ZNF833 | processed transcript |
| CNX | calnexin |
| GALC | galactosylceramidase |

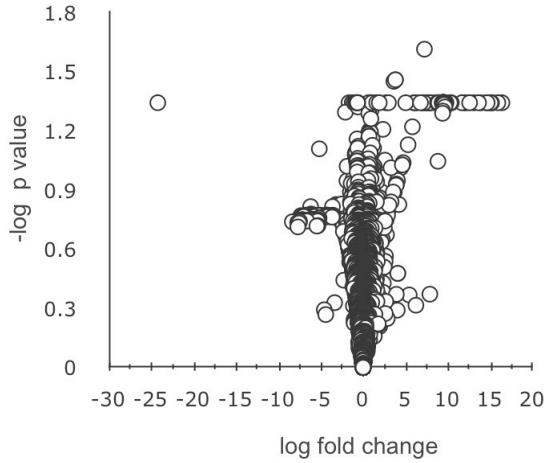

**Figure 1. Schematic representation using a volcano plot, of all the genes in the female group.** The plot was generated by plotting the -logarithm of the p-value (Y axis) vs. the logarithm of the fold change of the signal (X axis). p=0.05 is at y=1.3.

## Analysis of selected genes by Quantitative Real-Time PCR (Under SOW #1, 2 & 3)

To determine whether genes in the neural or oligodendroglia lineage were also expressed and/or deregulated in MS patients, we selected a group of genes in the array that matched that condition. We normalized the expression with respect to GAPDH and compared it to the QR-PCR using the same total RNA from pool female controls and MS females. The assay produced consistent results when compared to the microarray data. A summary of some of the genes analyzed is shown in figure 2.

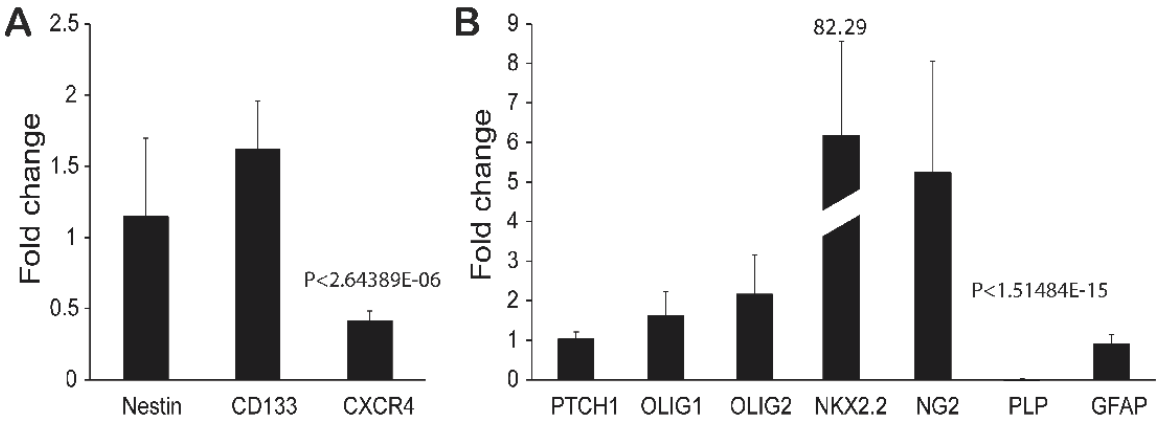


**Figure 2. Peripheral blood of twelve female RRMS patients showed to express some neural genes.** A, B) RNA was isolated from freshly drawn PBMCs and subjected to QRT-PCR using specific primers contained within exon sequences for the genes of interest. Samples were run in triplicates using an Applied Biosystems 7900HT-AB PCR machine. Fluorescent amplifications were measured and the fold change of gene expression over the control samples was calculated using the DCT method, with GAPDH as a housekeeping gene (GAPDH =1).

**Immunoblotting analysis (Under SOW # 2 & 3).** PBMCs pellets have been obtained from controls and MS patients and were being processed for immunoblotting analysis of expression for neural markers as proposed. A summary of some of the genes analyzed is shown in figure 3.

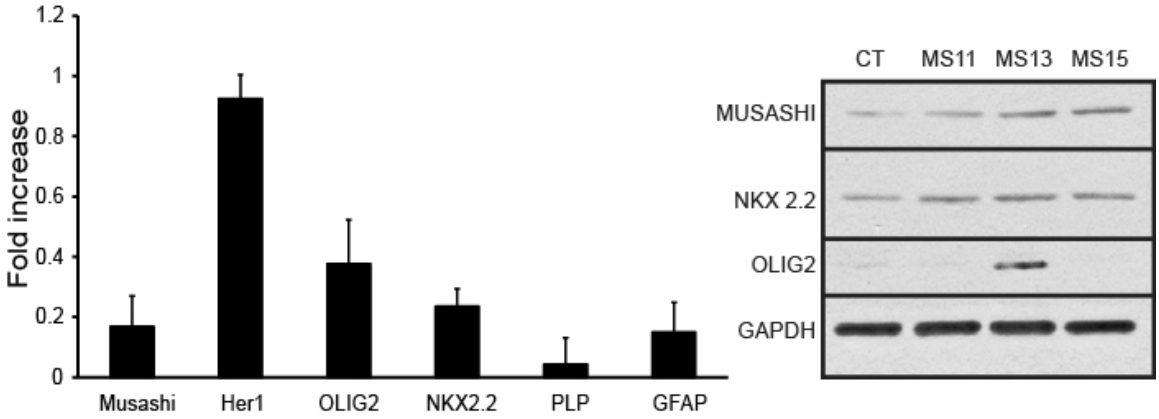


**Figure 3. Immunoblot analysis of neural gene expression in MSPBMCs.** PBMC cell pellets (5x106 cells) were lysed and used for SDS-PAGE/western blotting analyses. Twenty micrograms of total protein from one control and three RRMS patients were fractionated on a 4-20 % acrylamide SDS-gel and electrotransferred onto PVDF membranes. Blots were incubated with specific antibodies. Immunoreactions were developed using chemiluminiscent ECL reagents and films scanned for ImageJ analysis (NIH). Signals were normalized vs GADPH and expressed as fold increase respect to the control.

**Changes in Calnexin and Galactosylceramidase levels in blood cells from RRMS patients (Under SOW # 2 & 3).** Two interesting genes that were identified in our gene array analysis were calnexin (CNX) and Galactosylceramidase (GALC). We performed quantitative tests to measure for the abundance of mRNAs and proteins for both CNX and GALC. Quantitative real time polymerase chain reaction (qRT-PCR) analysis confirmed a significant reduction in the levels of CNX mRNA in a subset (n=7) of the RRMS mRNAs used for the wide genome analysis (Figure 4A). Furthermore, immunoblotting analysis confirmed a decrease in the abundance of the CNX proteins in total extracts of PBMCs from these RRMS patients. Figure 4B shows CNX protein levels by immunoblotting in just two different cases from the cohort of MS samples. These results confirmed that CNX expression is significantly lower than normal in PBMCs from RRMS patients. This may indicate that RRMS is compounded by a dysfunctional ER quality control apparatus, at least in PBMCs. Whether a similar defect is also present in myelin forming cells (OLs) in the brain and spinal cord of RRMS patients is presently unknown. Because CNX is one chaperone of fundamental importance for the production of correctly folded proteins, a reduction of CNX may lead to the introduction of errors during protein synthesis, transport and assembly, debilitating the mechanisms to maintain myelin during adult life.

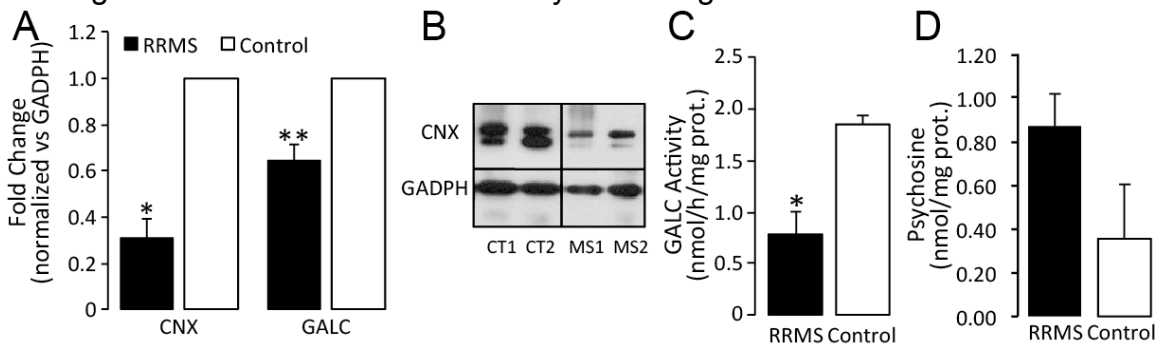

**Figure 4. Quantitative analyses of CNX and GALC levels in RRMS PBMCs.** A) Quantitative controls for CNX and GALC mRNA levels in RRMS PBMCs were performed using quantitative real time PCR. PBMC RNA was isolated from RRMS patients (n=7) and healthy volunteers (n=25). Real time PCR was performed using syber-green specific primers for human CNX and GALC and normalized for GAPDH. CNX and GALC levels in control PBMCs are set to =1. *p=0.0047; **p=0.00022 (Anova). B) Immunoblotting analysis for CNX in RRMS PBMCs. PBMCs from two control (CT1, CT2) and two RRMS (MS1, MS2) patients were analyzed using a specific anti-CNX antibody. GAPDH was used as loading control. C) GALC activity was measured in PBMCs isolated from RRMS donors (n=6) and healthy volunteers (n=4) by fluorometriy. *p=0.019 (Anova). D) Psychosine content was measured in lipid extracts prepared from total PBMCs (106 cell/ sample) from RRMS donors (n=3) and healthy volunteers (n=3) by liquid chromatography-mass spectrometry.

Analyses for GALC expression also confirmed our original finding in the gene array study. qRT-PCR analysis measured significant reductions of GALC mRNA in seven of the RRMS mRNAs used for the microarray analysis (Figure 4A). We measured the residual level of GALC enzymatic activity in protein extracts of RRMS PBMCs using a highly sensitive fluorometric method. Figure 4C shows quantitative data revealing a reduction of about 50% of GALC activity in RRMS PBMCs respect control values. Deficient GALC activity is known to lead to abnormal degradation of GALC substrates. To examine if RRMS PBMCs had altered levels of psychosine, we processed 3 of the RRMS PBMCs samples for lipid extraction and purification of psychosine. Psychosine levels were measured using LC-MS-MS, which provides an unparalleled level of sensitivity in the order of picomolar concentrations. Figure 4D shows LC-MS-MS quantitative data indicating increased levels of psychosine in RRMS PBMCs in comparison to control levels. These results indicate a defective catabolic pathway of the neurotoxin psychosine in PBMCs from RRMS. Because psychosine is a potent neurotoxin for OLs, accumulation of psychosine may also contribute as a compounding pathogenic factor during demyelination in RRMS.

**Analyses of cells with neural potential in the peripheral blood of RRMS patients (Under SOW # 2 & 3).** Total levels of CD133+ cells were quantified by flow cytometry. CD133+ cells were found significantly (*P*=0.033) decreased in our group of RRMS patients (0.17% ± 0.08%, N=20), when compared to controls (0.34% ± 0.27%, N=23) (Figure 5A). Interestingly, a gender effect appears to influence the abundance of CD133+ cells in RRMS. In controls, abundance of CD133+ cells in women (0.30% ± 0.20%, N=11) was not significantly different from that in men (0.38% ± 0.32%; N=12, *P*=0.74) (Figure 5B). In contrast, there was a significant (*P*=0.014) decrease in CD133+ cells in RRMS women (0.15% ± 0.07%, N=15) versus RRMS men (0.24% ± 0.05%, N=5) (Figure 5B). Control women had about twice the levels of CD133+ cell than RRMS women (*P*=0.034) while levels of CD133+ cells were not significantly different between control and RRMS men, (Figure 5B). Taken together, these results indicate a decrease in CD133+ cells in RRMS patients, which is more pronounced in affected women. In contrast, total levels of CD34+ cells were not affected in RRMS patients (Figure 5C) and no gender effect was observed (Figure 5D).

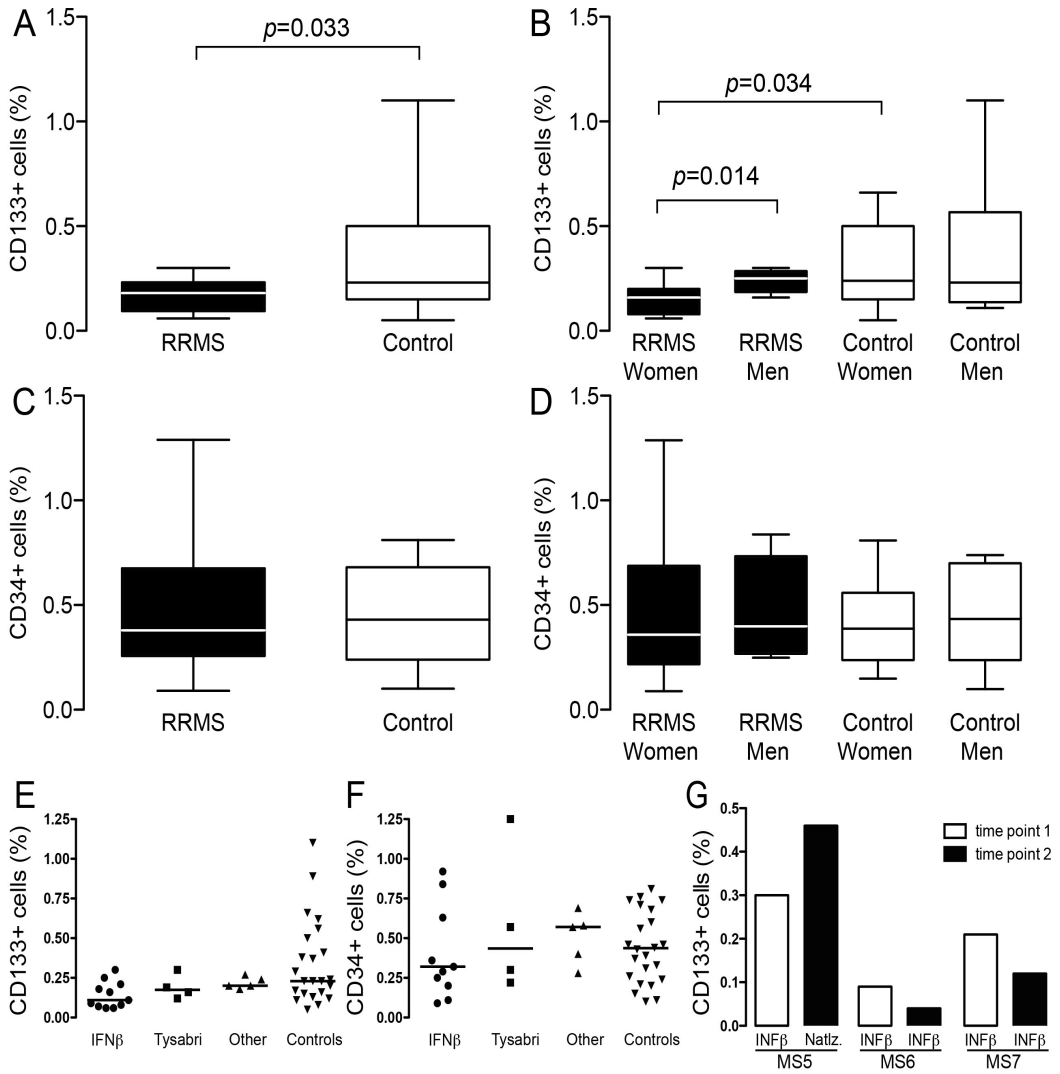


**Figure 5. Decreased levels of circulating CD133+ cells in RRMS patients.** Peripheral blood mononuclear cells from relapsing remitting multiple sclerosis (RRMS) patients and healthy volunteers were analyzed for the abundance of CD133+ and CD34+ cells by flow cytometry. **A)** Comparison between 23 healthy volunteers (Control), and 20 patients (RRMS) showed a significant decrease in CD133+ cells in RRMS patients (*P*=0.033). **B)** Comparison between genders (11 control women, 12 control men, 15 RRMS women and 5 RRMS men) showed significant decreases between RRMS women and men (*P*=0.014) and between control and RRMS women (*P*=0.034). **C)** Comparison between 23 healthy volunteers (Control), and 20 patients (RRMS) did not show significant differences in CD34+ cells. **D)** Comparison between genders (11 control women, 12 control men, 15 RRMS women and 5 RRMS men) also showed no difference in total levels of CD34+ cells. Values are expressed as the mean percentage ± standard error of CD34+ cells in the lymphocyte fraction. **E, F)** The percentage of CD133+ cells (E) and CD34+ cells (F) in the lymphocyte fraction from RRMS patients treated with interferon-β (IFN-β, N=11); natalizumab (Tysabri, N=4); glatiramer acetate, N=3; or no treatment, N=2 (Others) showed no significant difference among treatments on levels of either cell population (Kruskal-Wallis, *P*=0.15). **G)** The stability of circulating levels of CD133+ cells was assessed in three RRMS patients across 2 time points several months apart. MS5 patient (man) transitioned from interferon-β to natalizumab while MS6 (woman) and MS7 (man) patients remained on interferon-β. Bars represent percentages of CD133+ cells in the lymphocyte fraction.

**Changes in total CD133+ cells do not correlate with clinical variables and ongoing treatments in RRMS patients (Under SOW # 2 & 3).** Spearman analyses were performed to study for possible correlations between levels of peripheral circulating CD133+ cells and age of patients, EDSS, disease duration and time elapsed from last relapse. Table III shows these quantitative data. Although CD133+ cells were reduced

in RRMS patients, this reduction did not correlate directly with any of the clinical parameters. Levels of CD34+ cells correlated with time elapsed from last relapse (*P*=0.0177) but did not correlate with other clinical parameters (Table III).

We examined whether the treatments given to RRMS patients had any effect on the circulating levels of CD133+ or CD34+ cells. Total counts of CD133+ cells were not significantly different in patients treated with interferon-β (Avonex, Betaseron, Rebif), natalizumab (Tysabri), Glatiramer acetate or untreated (*P*=0.15), despite being significantly reduced in comparison to CD133+ cell levels in controls (Figure 5E). CD34+ cells were also non-significantly different in RRMS patients undergoing different treatments (Figure 5F). The stability of circulating levels of total CD133+ cells was assessed in three patients across 2 time points separated by 6 to 9 months (Figure 5G). CD133 levels fluctuated modestly and non-significantly across time. One patient transitioned from interferon-β to natalizumab therapy between the two-time point study and experienced a somehow larger and positive fluctuation of CD133+ cells but this was still not significant.

**Increased levels of CD34+CD133+ pPC in peripheral blood of RRMS patients (Under SOW # 2 & 3).** pPC are identified by their co-expression of cell surface markers CD133 and CD34 and are a significant source of rapid proliferating self-renewing pluripotential stem cells in a variety of tissues (Ratajczak et al., 2004). The participation of pPC in various diseases has also been reported, pointing to potential roles in regeneration and repair (Bühring et al., 1999). Little is known about the status of pPC in the blood of RRMS patients. Analysis of the subpopulation of CD133+ cells co-expressing CD34 showed a significant (*P*=0.0328) increase of pPC in the peripheral blood of RRMS patients within this study (Figure 6A). Furthermore, a significant (*P* =0.0226) correlation was found between the abundance of pPC in blood and the time elapsed since the last relapse (Figure 6B, Table III). RRMS patients showed higher levels of pPC immediately after or close to a relapse. Interestingly, we found that RRMS patients lost any correlation between the abundance of pPC and their age in contrast to controls (Figure 6C, 6D, Table III), underlining the possibility that relapses stimulate the levels of circulating pPC.

**RRMS and control CD133+ progenitors show similar hematopoietic and proliferative responses *in vitro* (Under SOW # 2 & 3).** We investigated for potential *in vitro* functional differences of CD133+ cells from RRMS patients. We first used a semi-solid methyl-cellulose assay and induced CD133+ cells to generate all distinct hematopoietic lineages in the presence of erythropoietin, stem cell factor, interleukin-3 and granulocyte macrophage colony stimulating factor. Although CD133+ cells from RRMS patients were capable of forming all expected colonies, we found no significant differences in colony morphology (Figure 6E-6H) and frequency (Figure 6I) with respect to colonies formed by CD133+ cells isolated from healthy donors. Potential differences in proliferative capacity of CD133+ cells were studied in serum-free medium formulated to stimulate the proliferation of stem cells. Although CD133+ cells from healthy donors responded significantly (*P*=0.0159) to the mitogenic stimulus, this proliferative response was not significant in RRMS cells (Figure 6J). Finally, we measured the *in vitro* response of CD133+ cells to migratory cues mediated by the SDF1-α-CXCR4 signaling

axis. For this, migration of CD133+ cells across a porous transwell filter was measured after stimulation with SDF1-α for 2 hours. We did not find significant differences in the migration response of RRMS CD133+ cells with respect to cells from control donors (Figure 6K).

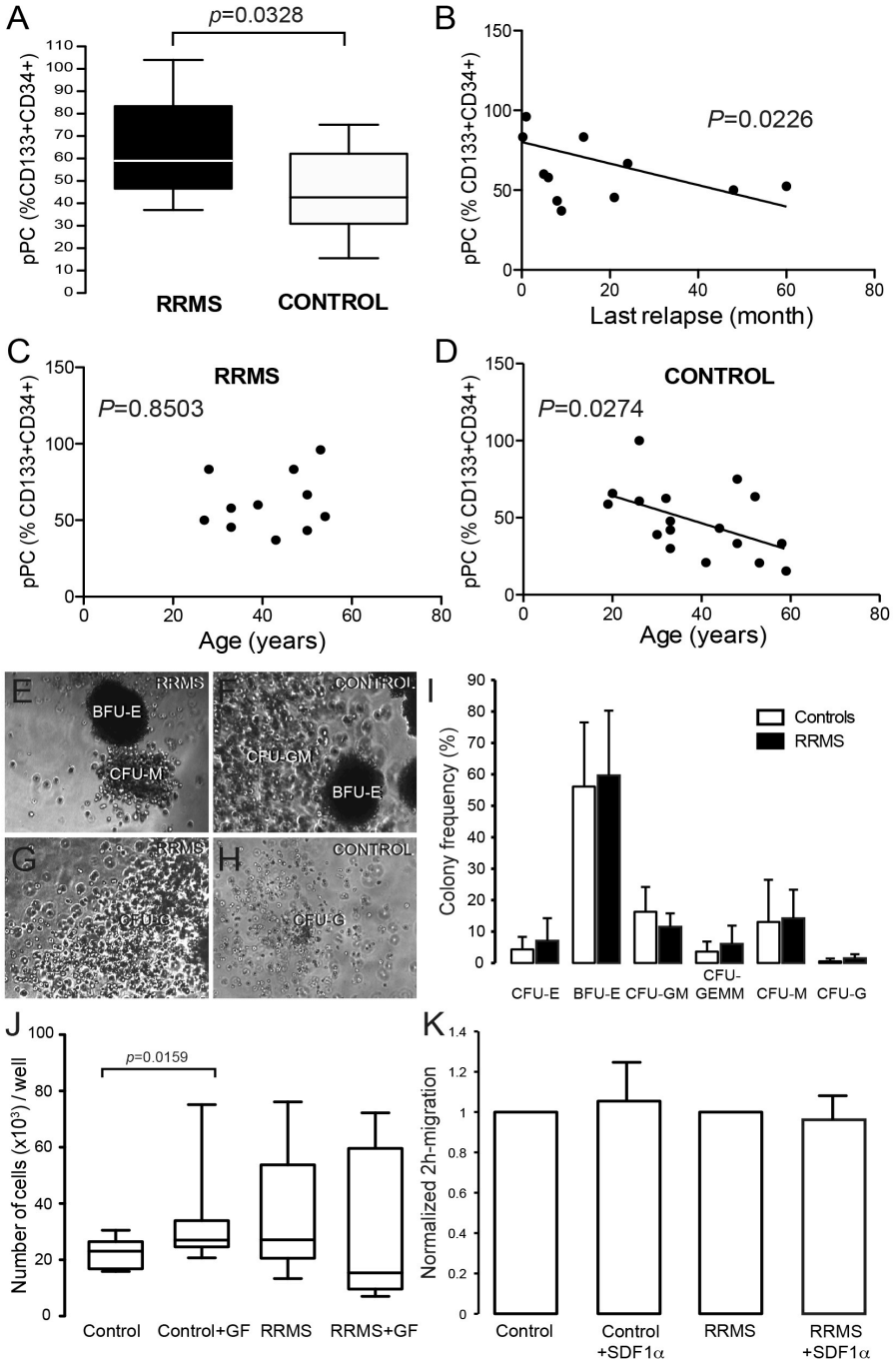


**Figure 6 (previous page). CD133+CD34+ pPC are increased in RRMS patients and correlate with relapses. A)** Peripheral blood mononuclear cells from relapsing remitting multiple sclerosis (RRMS) patients and healthy volunteers were analyzed for the abundance of double positive CD133/CD34 primitive progenitor cells (pPC) by flow cytometry. The percentage of CD133+CD34+ pPC was analyzed in 17 healthy controls and 13 RRMS patients. RRMS patients showed significantly higher numbers of double-stained CD133+CD34+ pPC (69.9% ± 27.4%) than controls (47.8% ± 22.2%, Mann-Whitney, *P*=0.0328). **B-D)** A correlation was found between the abundance of pPC in RRMS patients and time elapsed since the last relapse (B, *P*=0.0226) but not with the age of the patient (C). Instead,

pPC levels correlated with age in control volunteers (D) (*P*=0.0274). **E-H)** Representative images of colonies after culturing CD133+ cells isolated from 3 relapsing remitting multiple sclerosis (RRMS) patients (E,G) and 3 control (F,H) individuals in Methocult medium for 15 days. All photographs were taken at 100X magnification. **I)** Colonies were counted directly from each culture, and the results grouped according to their morphological features. CFU: colony forming unit; CFU-E: erythrocyte; BFU-E: burst-forming unit erythrocyte; CFU-GM: granulocyte monocyte; CFU-GEMM: granulocyte erythrocyte monocyte megakaryocyte; CFU-M: monocyte; CFU-G: granulocyte. Colony frequency is expressed as a percentage of each type of colony of the total of colonies formed ± standard error. No significant differences were determined. **J)** The total number of cells responding to growth factors (GF) was counted six hour after plating CD133+ cells ($3x10^4$ cells per well) in serum-free medium with or without the addition of GF. Significant proliferation (*P*=0.0159) was measured in control CD133+ cells but not in RRMS cells. **K)** Transwell migration was measured two hours after plating CD133+ cells ($3x10^4$ cells per well) pre-labeled with Calcein AM and incubated with or without the addition of stromal cell-derived factor-1$\alpha$ (SDF-1$\alpha$). No significant migration differences between RRMS and control cells were measured. All experiments were performed in 3 independent experiments (3 RRMS and 3 controls) in triplicates.

## Table II. Demographics of 20 RRMS patients for flow cytometry

| Patient Code | Gender | Age years | EDSS | Treatment Chemical names | Duration years |
|---|---|---|---|---|---|
| MS03 | Female | 47 | 0.5 | Interferon Beta | 10 |
| MS04 | Female | 46 | 6 | Interferon Beta | 7 |
| MS05 | Male | 50 | 7 | Interferon Beta | 14 |
| MS06 | Female | 50 | 6.5 | Interferon Beta | 6 |
| MS07 | Male | 54 | 0 | Interferon Beta | 5 |
| MS08 | Female | 39 | 5.5 | Glatiramer Acetate | 11 |
| MS09 | Male | 43 | 0.5 | Glatiramer Acetate | 6 |
| MS10 | Male | 53 | 5 | Interferon Beta | 5 |
| MS11 | Female | 33 | 0 | Natalizumab | 3 |
| MS12 | Female | 46 | 6 | Interferon Beta | 15 |
| MS13 | Female | 33 | 1 | Interferon Beta | 9 |
| MS14 | Female | 28 | 3 | Interferon Beta | 1 |
| MS15 | Female | 33 | 2 | None | 0.25 |
| MS16 | Female | 27 | 4.5 | Natalizumab | 7 |
| MS18 | Female | 55 | 1 | Glatiramer Acetate | 0.33 |
| MS19 | Female | 30 | 4.5 | Interferon Beta | 2 |
| MS20 | Female | 54 | 5 | Natalizumab | 15 |
| MS21 | Female | 38 | 0 | None | 1 |
| MS22 | Male | 46 | 6.5 | Interferon Beta | 25 |
| MS23 | Female | 38 | 1 | Natalizumab | 7 |
| MS24 | Female | 48 | 1 | Natalizumab | 2 |
| MS25 | Male | 34 | 4.5 | Natalizumab | 8 |
| MS26 | Male | 48 | 2 | Natalizumab | 22 |
| MS26 | Male | 48 | 2 | Natalizumab | 22 |
| MS28 | Male | 53 | 3 | Natalizumab | 5 |
| MS29 | Female | 51 | 0 | Natalizumab | 3 |
| MS30 | Male | 30 | 1 | Natalizumab | 8 |
| MS31 | Male | 34 | 4.5 | Natalizumab | 4 |
| MS32 | Male | 47 | 0.5 | Natalizumab | 14 |

## Table III. Correlation with Clinical Parameters

**AGE OF RRMS GROUP**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| ***#XY pairs*** | 20 | 20 | 13 | 13 |
| ***Spearman "r"*** | 0.2833 | 0.3573 | 0.1842 | 0.05817 |
| ***p value (two-tailed)*** | 0.2262 | 0.122 | 0.5469 | 0.8503 |

**EDSS**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| ***#XY pairs*** | 20 | 20 | 13 | 13 |
| ***Spearman "r"*** | -0.06624 | -0.01134 | 0.24 | 0.1972 |
| ***p value (two-tailed)*** | 0.7814 | 0.9622 | 0.4297 | 0.5183 |

**DISEASE DURATION (DD)**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| ***#XY pairs*** | 20 | 20 | 13 | 13 |
| ***Spearman "r"*** | -0.0839 | 0.1279 | 0.189 | -0.1848 |
| ***p value (two-tailed)*** | 0.7251 | 0.5909 | 0.5364 | 0.5455 |

**EDSS/DD**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| ***#XY pairs*** | 20 | 20 | 13 | 13 |
| ***Spearman "r"*** | -0.09585 | -0.03768 | -0.05241 | 0.4221 |
| ***p value (two-tailed)*** | 0.6877 | 0.8747 | 0.865 | 0.1508 |

**TIME FROM LAST RELASPSE**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| ***#XY pairs*** | 20 | 20 | 13 | 13 |
| ***Spearman "r"*** | -0.0302 | -0.5239 | 0.707 | -0.6107 |
| ***p value (two-tailed)*** | 0.8994 | **0.0177** | **0.0069** | **0.0226** |

**AGE OF CONTROL GROUP**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| ***#XY pairs*** | 20 | 20 | 13 | 17 |
| ***Spearman "r"*** | -0.1213 | -0.2624 | 0.6363 | -0.5335 |
| ***p value (two-tailed)*** | 0.5813 | 0.2265 | **0.006** | **0.0274** |

## Conclusions of the analysis of CD133 precursors in RRMS peripheral blood.

We analyzed the circulating levels of bloodborne stem cells identified by their expression of CD133 and CD34 surface markers in a cohort of 20 RRMS patients. Our analyses showed a significant reduction of total levels of CD133+ cells with no significant change in the levels of CD34+ cells. However, the pPC subpopulation of stem cells identified by the co-expression of CD133 and CD34 markers were significantly increased in RRMS patients, particularly in those that underwent a recent relapse. These results suggest that levels of CD133+ cells and particularly those of CD133+CD34+ pPC may help identify RRMS patients who may be close to a relapse or with higher risk of developing a relapse.

The RRMS patients who volunteered for this study had first line and second line medications, including IFN-β, natalizumab, and Glatiramer Acetate. For the last twenty years, most RRMS patients start a treatment immediately following diagnosis. Hence, the availability of untreated RRMS patients was scarce to null at the time of enrollment in our study. Non-treated MS patients exist among the progressive forms of the disease. However, these were not included in our study because progressive forms (primary and secondary) are different than RRMS (Arnason, 2011), which would have introduced biased results on their CD133+ cells status when comparing with RRMS patients.

CD133+ stem cells circulate in minute numbers but are pluripotent. Active research aims to study their function in an array of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, especially for their angiogenesis regenerative properties. In MS, CD133+ stem cells are of interest for both hematopoiesis and neurogenesis. CD133 is a pentaspan protein expressed in stem cells from a variety of tissues, including hematopoietic (Miraglia et al., 1997; Yin et al., 1997) and neural cells (Porat et al., 2006). As such, CD133+ have been isolated from the mouse and human brain and showed their ability to generate neurons, oligodendrocytes and astrocytes. Because CD133 expression marks also a circulating population of pluripotential stem cells in the blood, the possibility that bloodborne CD133+ stem cells may participate in demyelination-remyelination in MS cannot be excluded. The characterization of CD133+ populations in this study is a first step towards understanding the potential role of these cells in MS.

Our results showed a significant reduction of the total levels of CD133+ cells in the blood of RRMS patients. The reduction of CD133 levels may represent a reduced proliferation of these cells. For example, IFN-β affects the *in vitro* proliferation of peripheral mononuclear cells (Noronha et al., 1993). Patients under IFN-β treatment have shown decreased numbers of newly produced T lymphocytes and the association between reduced thymic output and low peripheral T lymphocytes can be a cause of leukopenia, a frequent side effect of the IFN-β therapy (Zanotti et al., 2011). Because about half of the patients in this study were under IFN-β treatment, this possibility cannot be completely excluded. However, our comparison of the CD133+ levels between IFN-β-treated patients, natalizumab-treated patients, and a heterogeneous group treated with Glatiramer Acetate or no treatment showed no significant differences among the various groups of RRMS patients. This suggests that the decrease in blood levels of CD133+ cells is likely a feature of these patients. Furthermore, the absence of significant changes in CD34+ cells also points to the absence of a generalized repression by IFN-β. However, other factors may have contributed to lower the levels of bloodborne CD133+ cells in RRMS patients, namely (i) CD133+ cells may undergo higher rates of cell death in RRMS; (ii) CD133+ cells may have lower or slower proliferation rates, leading to low amplification of the circulating pool; (iii) CD133+ cells may migrate from blood into solid tissues such as the CNS and (iv) CD133+ cells may differentiate more rapidly into hematopoietic and/or other cell lineages. It is tempting to speculate that CD133+ cells may be recruited to repair demyelination; however it is not yet known whether CD133+ cells can directly engage in myelin repair in MS. One working hypothesis is that MS patients undergo an active phase of remyelination, during which bloodborne stem cells could contribute directly or indirectly to the repair mechanism. Several reports support the potential of CD133 cells to form neural cell

types. For example, human fetal CD133+ neural stem cells and human CD133+ stem cells mobilized from peripheral blood promoted faster recovery after transplantation in a rodent model of spinal cord injury (Sasaki et al., 2009). Similarly, CD133+ cells improve peripheral nerve regeneration (Kijima et al., 2009). Amyotrophic lateral sclerosis patients grafted with autologous CD133+ cells in the frontal motor cortex showed some improvement in quality of life and life expectancy (Martinez et al., 2009). Our results provide initial evidence that at least the hematopoietic potential of RRMS CD133+ cells was not significantly compromised in affected patients, which would suggest that their neural potential is also intact. Overall, these reports underline the potential role for CD133+ cells in neural repair. Whether CD133+ cells from peripheral blood could be used for improving repair in RRMS brains and whether CD133+ cell-mediated neural repair occurs *in vivo* in the MS brain need further studies.

Our study found that the reduction of CD133+ cells was more pronounced in RRMS women than men. Lower ratios of circulating cells co-expressing CD133 and CD34 have been reported in healthy women than in men (Reichelt et al., 2009). Reduced levels of CD133+ cells in RRMS women may be related to the higher incidence of RRMS in women (3:1) (Compston and Coles, 2008). For example, women affected by the autoimmune disease systemic lupus erythematosus are also characterized by a decrease in circulating CD133+ cells when compared to their healthy counterparts (Moonen et al., 2007). In addition, a larger pool of CD133+ cells in men may be related to sex hormones. Interaction between progesterone and estrogen and stem cells has been described (Brannvall et al., 2002; Hong et al., 2004). Little is known about the status of CD133+ cells in RRMS patients. Hence, to our knowledge, our study is the first to report that circulating CD133+ cells may be a relevant variable correlating gender effects in MS. As such, a larger study of the circulating levels of CD133+ cells in MS patients should be explored to determine its use as a screening tool for patients at risk of developing MS.

Correlation studies between abundance of circulating CD133+CD34+ pPC and age are somewhat controversial (Heiss et al., 2005; Shaffer et al., 2006; Reichelt et al., 2009). Aging was shown to decrease circulating CD34+ cells, but not those of circulating CD133+ cells in subjects around 60 year-old (Povsic et al., 2010). In our study, we found a significant correlation of pPC levels with age in the control group. Interestingly, this correlation was lost in patients with RRMS, likely indicating the immune response of these patients in response to relapses. It remains to be seen whether progressive MS patients have the same CD133 variability.

The regeneration potential intrinsic for CD133+ stem cells and for pPC in particular is of high relevance. For example, CD133+ cells have the ability to generate both hematopoietic and endothelial progenitors (Gehling et al., 2000). These cells, which also express the CXCR4 receptor, respond to SDF-1$\alpha$ and VEGF and are thought to participate in angiogenesis (Pozzobon et al., 2010; Adini et al., 2013). CD133+ stem cells and pPC are also responsive in a variety of pathological conditions including cancers, vascular and cardiac damage, liver cirrhosis and Duchenne dystrophy (Turan et al., 2007; Marchesi et al., 2008; Gehling et al., 2010; Pescini et al., 2010). Our study found a positive correlation between the abundance of circulating pPC in RRMS patients and relapse, with higher levels of pPC during recent clinical episodes. Levels of pPC in our group of healthy donors is similar to previous reports (Moonen et

al., 2007). An increase in circulating levels of pPC in patients with recent relapses may be relevant to promote peripheral and central tissue repair. Alternatively, pPC may be mobilized to replenish the pool of hematopoietic cells infiltrating the CNS during a relapse. Their involvement in these processes requires further investigation.

In summary, the results presented here demonstrate differences in the circulating population of CD133+ cells in RRMS patients. Our findings of levels of CD133+ cells lower in RRMS women than in RRMS men may be of relevance to understand gender differences in RRMS patients. Increases in pPC levels in relapsing RRMS patients may be another indicator of disease status and recovery.

**In vitro and in vivo analyses of neurogenic differentiation of blood derived precursors (Under SOW #3).** CD133+ cells were isolated from control and RRMS blood and used to test their neurogliogenic potentia;. To culture the CD133+ cells we used a modified medium for blood stem cells described for CD133 cell cultures. This medium, named HSCM, consists of Isocove's modified Dulbecco´s medium (IMDM) supplemented with 10% FBS, 100 ng/ml Flt-3, 20 ng/ml IL-6, and 100 ng/ml SCF. We found that when cultured in this medium, CD133 cells survived well and proliferated for the first 3-4 passages (figure 7). However, after the fourth passage, CD133+ cells slowed their proliferation rate and eventually stopped dividing.

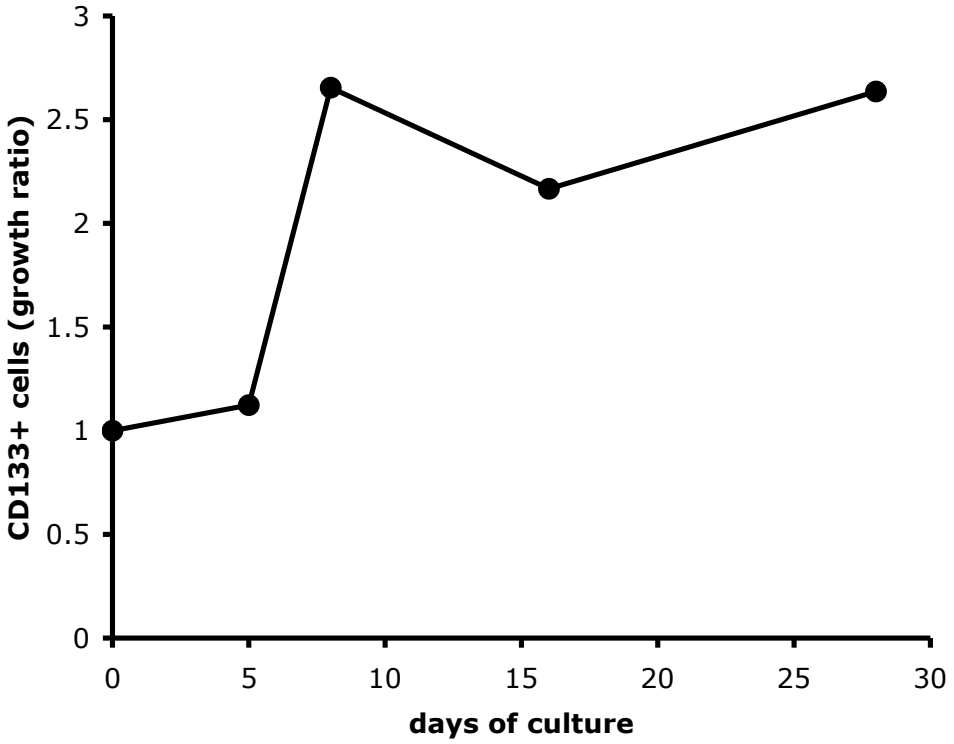


**Figure 7: Proliferation curve of CD133+ stem cells isolated from total mononuclear blood fractions. Data represents the growth ratio of CD133+ cells at each passage normlized vs the original input.**

These results suggest that HSCM promotes early proliferation of isolated CD133+ stem cells but it does not sustain their long-term proliferation. This indicated that our medium

lacks essential factors to promote cell division. Additionally, this lack of proliferation may be caused by premature differentiation of CD133+ stem cells into more mature post-mitotic cells under the culture conditions. We dedicated a significant amount of time and resources to developing a culture media based on the formulation of HSCM, which will facilitate long-term proliferation.

For this, we tested the effect of various factors on proliferation of CD133+ cells after passage 4 (a passage where initial proliferation is stalled). Factors were supplemented to the HSCM base medium and compared to the HSCM control condition. We tested the effect of exogenous additions of 100 ng/ml of stem cell factor (SCF) on the survival and viability of CD133+ stem cells. For this, CD133+ stem cells were split at passage 4 and 25,000 cells were seeded on new wells in HSCM with and without the addition of SCF. Figure 8 shows numbers of CD133+ cultured for 7 days. Cells in HSCM alone showed a reduction of about 20% of CD133+ cells. Instead, the survival of CD133+ cells was >100% when SFC was supplemented to HSCM. When SFC was added to our regular neural stem cell medium (NSCM), its survival effect was not observed. CD133+ cells maintained in NSCM with ot without SFC failed to survive after one week (Figure 8).

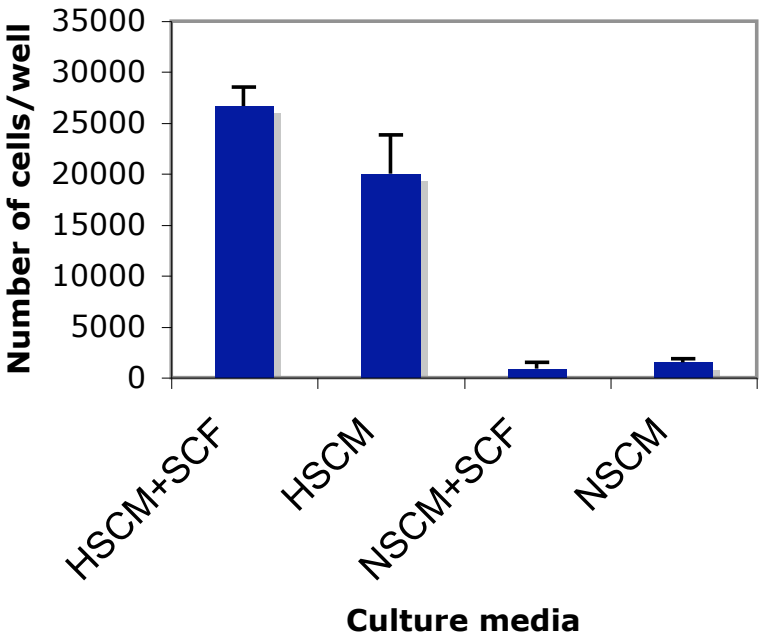


**Figure 8: Effect of stem cell factor on the survival/proliferation of CD133+ stem cell. Data represents absolute numbers of CD133+ cells one week after culture in HSCM, HSCM supplemented with 100ng/ml of SFC, NSCM and NSCM supplemented with 100 ng/ml of SCF. Original input of cells was 25,000 CD133+ cells per well.**

Figure 9 shows representative random pictures from CD133 stem cells grown in each medium. CD133 cells grown in HSCM in the presence of SFC showed well defined, healthy morphologies.

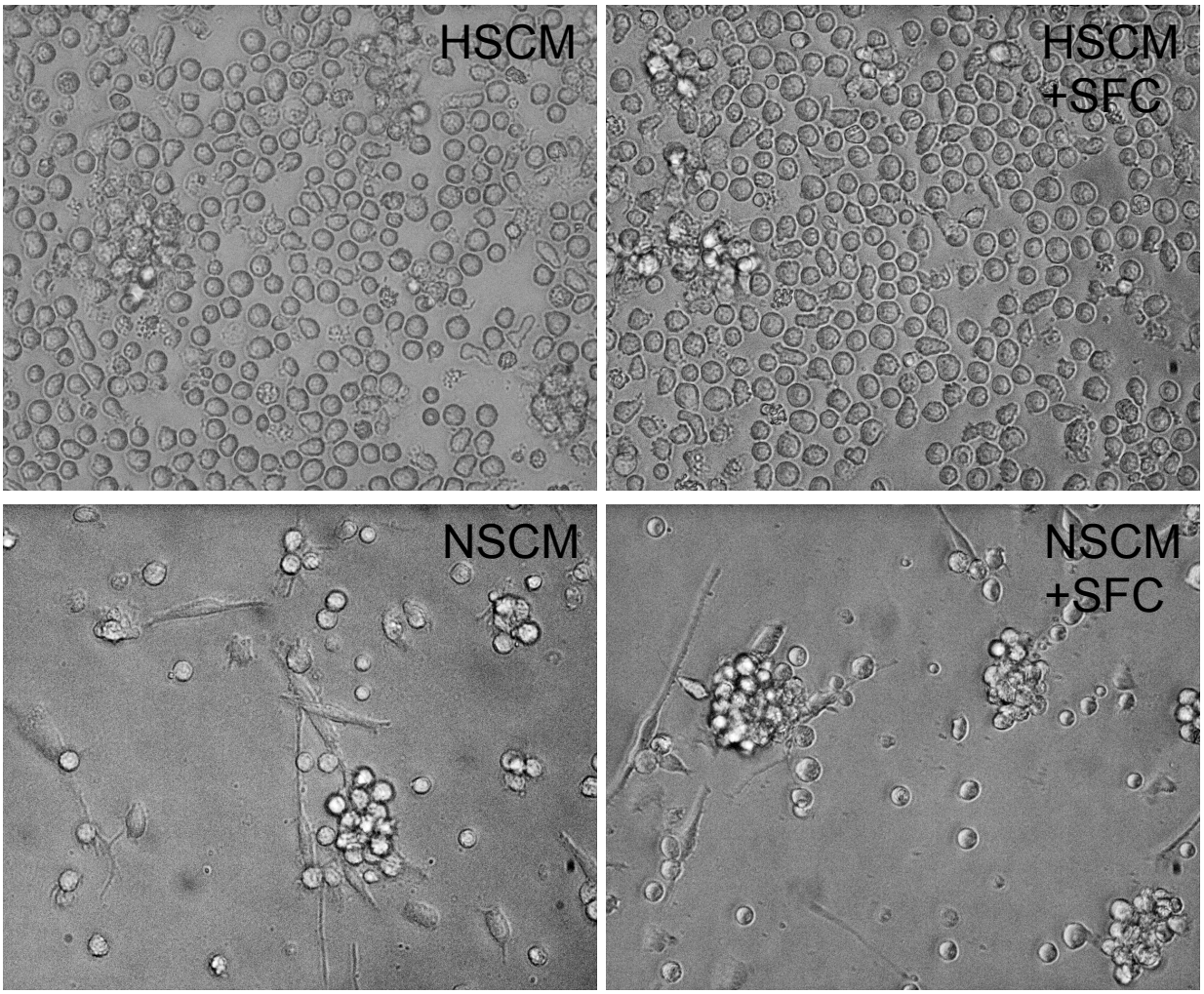


**Figure 9: Effect of stem cell factor on the survival/proliferation of CD133+ stem cell. Pitcures were taken 7 days after culture of CD133+ cells in HSCM, HSCM supplemented with 100ng/ml of SFC, NSCM and NSCM supplemented with 100 ng/ml of SCF.**

Further experiments were conducted using a variety of facgtors inclduing Flt3, IL6, IL3, SCF. None of these combinations rendered a condition that would support long-term maintenance of precursors. We also found that basic neural stem cell medium (BSCM) used for growing brain-derived neural stem cells in neurospheres was not useful. We formulated a human neural stem medium (HNSM), which is composed by DMEM/F-12 supplemented with 20 ng/ml EGF, 10 ng/ml bFGF and 1ng/ml heparin, and 10% FBS. Using this medium, we observed the formation of colony-like structures after 6-8 days in culture. However, these colonies proliferated at a very slow rate. Our final conclusion from these experiments was that the percentage of stem cells with true potential to differentiate in neural cells circulating in the blood is extremely low for their in vitro culture and expansion. We suspected that neurogenic differentiation -if any- might

require in vivo microenvironments that were not supported by the in vitro conditions. This was analyzed using the mutant Shiverer as proposed. The shiverer mouse undergoes dysmyelination and has been used for testing the as a host for transplantation of CD133 cells. Blood-derived CD133+ precursors isolated from RRMS patients (we tested 6 different preparations) were transplanted with DiI-labeled cells from healthy and RRMS donors. Transplanted mice were allowed to survive for up to 20 days before immunohistochemical analysis. While transplanted cells were identifiable, we found no evidence that these cells differentiated into neuronal or glial lineages. While our analyses did not show neural commitment in the Shiverer model, we still hold the possibility that other models with neuroinflammatory components may be suitable to investigate this possibility. As such we are planning to study CD133+ behavior in animals under experimental allergic encephalomyelitis.

## CONCLUSION

Our experiments determined that circulating CD133+ cells are below normal values in the blood of many RRMS patients. Interestingly, the fraction of CD133+ cells that co-express CD34 is elevated in MS patients closer to a relapse, suggetsing that these may be involved in events following an attack (e.g. remyeliantion). The significance of this finding needs further examination. In vivo experiments are being planned to characterize the properties of these cells further in a model of demyelination that undergoes inflammation, which may be more suitable to test their neurogenic potential. Our gene analysis confirmed the expression of various neural genes in MS blood, albeit the cells that express these genes could not be mainatained in vitro for more than a few weeks. Further analysis of the significance of these findings is being planned. Finally and importantly, our study identified several genes which are deregulated in MS. Two of these, calnexin and galactosylceramidase, are of high interest because they are associated with demyelinating conditions. Further evaluation of their relevance in MS is currently being analyzed.

## KEY RESEARCH ACCOMPLISHMENTS

1) Abundance of CD133+ was found lowered in peripheral blood from RRMS patients.
2) Abundance of CD133+CD34+ precursors was increased in peripheral blood from RRMS patients.
3) Gene array analyses of PBMCs from MS and healthy controls have been done and yielded very significant and large volume of data. New research lines were derived from this project (see items 4 and 5 below)
4) ER chaperone protein calnexin was found decreased in blood cells from many patients. This decrease may bear relevance in immune responses in these patients.
5) Galactosylceramidase was also found decreased in the blood of RRMS patients, along with modest increases of a neurotoxin, psychosine.

## REPORTABLE OUTCOMES

1) A repository of cells and plasma from RRMS patients is available in our laboratory.
2) Part of this work supported a funding request to the National Multiple Sclerosis Society. A grant was awarded.
3) Part of this work supported a funding request to DoD. This application was not awarded.
4) Kaminska-Czajkowska, A, Finkielsztein, A. Boullerne, A. Givogri MI, Castelvetri L, Skias D, Hartman E, Balabanov R, Feinstein D, Bongarzone ER. Expression of sonic hedgehog targeted genes in peripheral blood mononuclear cells of patients with multiple sclerosis. Society for Neuroscience. Washington. nov, 2011
5) A, Finkielsztein, Kaminska-Czajkowska, A, A. Boullerne, A. Givogri MI, Ulloa V, Skias D, Hartman E, Balabanov R, Feinstein D, Bongarzone ER. Reduction of cd133 positive stem cells circulating in the peripheral blood of patients with relapsing remitting multiple sclerosis. Society for Neuroscience. Washington. nov, 2011
6) A, Finkielsztein, Kaminska-Czajkowska, A, A. Boullerne, A. Givogri MI, Ulloa V, Skias D, Hartman E, Balabanov R, Feinstein D, Bongarzone ER. . A role for cd133 positive stem cells in patients with relapsing remitting multiple sclerosis. American society for neurochemistry. Baltimore, march 2012.
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8) A, Finkielsztein, Kaminska-Czajkowska, A, A. Boullerne, A. Givogri MI, Ulloa V, Skias D, Hartman E, Balabanov R, Feinstein D, Bongarzone ER. Bloodborne cd133/cd34 primitive progenitor cells increase after relapses in multiple sclerosis. Manuscript in review. Journal of Neurochemistry.

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## APPENDICES

None

**SUPPORTING DATA**

Data has been embedded within the narrative.

NEUROSCIENCE 2011

Presentation Abstract

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| Program#/Poster#: | 322.13 |
| Presentation Title: | Expression of sonic hedgehog targeted genes in peripheral blood mononuclear cells of patients with Multiple Sclerosis. |
| Location: | 152A |
| Presentation time: | Monday, Nov 14, 2011, 11:00 AM -11:15 AM |
| Authors: | ***A. KAMINSKA-CZAJKOWSKA**[1,4], A. FINKIELSZTEIN[1], A. BOULLERNE[2], M. GIVOGRI M.I[1], L. CASTELVETRI[1], D. SKIAS[3], E. HARTMAN[3], R. BALABANOV[4], D. FEINSTEIN[2], E. BONGARZONE[1];<br>[1]Anat. and Cell Biol., [2]Dept. of Anesthesiol., [3]Neurol. and Rehabil. Med., Univ. of Illinois At Chicago, Chicago, IL; [4]Dept. of Neurol., Rush Univ., Chicago, IL |
| Abstract: | Neural stem cells (NSCs) are a subtype of immature, proliferating cells in the nervous system that can self - renew and generate both neurons and glia. Adult |

NSCs have been found in the two principal adult neurogenic regions, the hippocampus and subventricular zone (SVZ) and in some non-neurogenic regions, including spinal cord. Recent findings have shown that peripheral blood, cord blood and bone marrow could generate, under appropriate circumstances, stem cells with neural potential.
In this study we have challenged the idea that cells with neural properties may circulate in the blood of patient suffering of Relapsing Remitting Multiple Sclerosis (RRMS). Multiple sclerosis (MS) is a chronic inflammatory disease that leads to axonal loss due to recurrent episodes of immune - mediated multi-focal demyelination.
Our work recruited 16 patients with RRMS and 23 healthy controls. Peripheral Blood Mononuclear Cells (PBMCs) were isolated through gradient of ficoll- paque and used for RNA and protein analyses. Gene array hybridization showed up-regulation of various components of the Sonic hedgehog (Shh) pathway including, Olig1 and Olig2.
Taken together, our results suggest that PBMCs from RRMS patients contain a subpopulation of cells expressing neuronal genes. The functional role of these cells in disease is discussed

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|---|---|
| Disclosures: | **A. Kaminska-Czajkowska:** None. **A. Finkielsztein:** None. **A. Boullerne:** None. **M. Givogri M.I:** None. **L. Castelvetri:** None. **D. Skias:** None. **E. Hartman:** None. **R. Balabanov:** None. **D. Feinstein:** None. **E. Bongarzone:** None. |
| Keyword(s): | NEURAL STEM CELLS |
| | MULTIPLE SCLEROSIS |
| | OLIGODENDROCYTE |
| Support: | DOD PR080601 |

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| Location: | 152A |
| Presentation time: | Monday, Nov 14, 2011, 8:00 AM - 8:15 AM |
| Authors: | ***A. FINKIELSZTEIN**[1], A. CZAJKOWSKA-KAMINSKA[1], A. BOULLERNE[2], M. I. GIVOGRI[3], V. ULLOA[3], D. SKIAS[3,4], E. A. HARTMAN[4], R. D. BALABANOV[5], D. FEINSTEIN[2], E. R. BONGARZONE[1];<br>[1]Anat. and Cell Biol., [2]Dept. of Anesthesiol., [3]Dept. of Anat. and Cell Biol., Univ. of Illinois At Chicago, Chicago, IL; [4]Neurol. and Rehabil. Med., Univ. of Illinois at Chicago, Chicago, IL; [5]Rush Univ., Chicago, IL |
| Abstract: | Multiple Sclerosis (MS) is a serious condition that affects both women and men, |

which it is characterized by an accelerated and sometimes stable loss of myelin-producing cells (oligodendroglia) of the Central Nervous System (CNS). Although the present knowledge of the disease suggests there is no cure for MS, renewed hope has now risen from the potential stem cell therapies. In particular, the peripheral blood mononuclear cells (PBMCs) are the targets of intensive research aimed at characterizing its stem cell properties and their potential to generate neurogliogenic cell types such as oligodendroglia, which could potentially replace the damaged caused to the CNS in MS patients.

To start testing this in more detail, we have undertaken a major study of the abundance of stem cells mobilized in the blood of patients undergoing relapsing remitting MS (RRMS). A total of 23 patients and 25 healthy controls were recruited for this study. PBMCs were isolated and stem cells were analyzed for their expression of the CD133 stem cell marker. Patients were grouped by gender and comparisons of CD133+ cells were performed after fluorescent activated cell sorting (FACS) analysis. CD133+ cells were analyzed in both lymphocytic and monocytic fractions from RRMS PBMCs and compared with that from healthy controls. Using 23 controls and 21 RRMS patients as blood donors, we found a significant negative strong correlation between the abundance of CD133+ cells in both the monocytic and lymphocytic fractions and the MS condition. Examination of gender differences, however, showed that while there is a significant difference between controls and RRMS in lymphocytes in both genders, there is only a marked trend in monocytes, which does not reach significance, probably due to the sample size.

Further studies are now being conducted to determine the growth, self-renewal and differentiating capacity of the CD133+ cells from MS and controls. We believe these studies will establish the groundwork to improve our understanding of MS and increase the odds for the cure of MS.

| | |
|---|---|
| Disclosures: | **A. Finkielsztein:** None. **A. Czajkowska-Kaminska:** None. **A. Boullerne:** None. **M.I. Givogri:** None. **V. Ulloa:** None. **D. Skias:** None. **E.A. Hartman:** None. **R.D. Balabanov:** None. **D. Feinstein:** None. **E.R. Bongarzone:** None. |
| Keyword(s): | STEM CELL |

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| | BLOOD |
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## A ROLE FOR CD133 POSITIVE STEM CELLS IN PATIENTS WITH RELAPSING REMITTING MULTIPLE SCLEROSIS

**A. FINKIELSZTEIN**[1], A. CZAJKOWSKA-KAMINSKA[1], A. BOULLERNE[2], M. I. GIVOGRI[3], V. ULLOA[3], D. SKIAS[3,4], E. A. HARTMAN[4], R. D. BALABANOV[5], D. FEINSTEIN[2], E. R. BONGARZONE[1];
[1]Anat. and Cell Biol., [2]Dept. of Anesthesiol., [3]Dept. of Anat. and Cell Biol., Univ. of Illinois At Chicago, Chicago, IL; [4]Neurol. and Rehabil. Med., Univ. of Illinois at Chicago, Chicago, IL; [5]Rush Univ., Chicago, IL

BACKGROUND: Multiple Sclerosis (MS) is a demyelization disease that affects both men and women of all geographic areas of the planet and of various genetic backgrounds. MS is not associated with any particular mutation and their cellular features are not clearly understood. One type of MS, Relapsing Remitting MS (RRMS), is characterized by cycles of acute attacks, followed by remissions, where there is active myelin repair. It is hypothesized that pluripotent cells carrying a CD133 marker may be involved in the repair process.
RESULTS: We tested 20 MS and 23 healthy controls and found that women affected by MS have a decreased number of CD133+ Hematopoietic Stem Cells in peripheral blood when compared to healthy controls, while men have no difference in the same cell population. We also found that CD34+ cells in MS women is, surprisingly, elevated while CD133+ is decreased with respect to MS men. CD133+ cells from controls and MS had the same chemotactic response to SDF-1$\alpha$ in a chemotaxis assay and produced the same type and frequency of HSC differentiated colonies in response to EPO, SCF, IL-3, and GM-CSF. However, cells from MS patients showed a lower proliferative response to FLTK3 ligand, SCF and IL-3 in a proliferation assay.
SIGNIFICANCE: Blood-borne CD133+ stem cells may have neuroprotective capacity during the remission phase of the RRMS that may be more affected in women with MS than in men with MS. This difference between CD133+ fractions in women and men may key to understanding the differences in the number of affected women and men in the population and may impact on future MS treatments.

Expression of neurogliogenic genes Olig1 and Olig2 in Peripheral Blood Mononuclear Cells in from Patients with Relapse Remitting Multiple Sclerosis.

*Czajkowska-Kaminska A.[1], Finkielsztein A.[1], Boullerne A.[2], Givogri M.I.[1], Cantuti L.[1], Skias A.[3], Hartman E.[3], Balabanov R.[4], Feinstein D.[2], Bongarzone E.R.[1]*

1-Dept. of Anatomy and Cell Biology, University of Illinois at Chicago, 2-Dept. of Anesthesiology and 3-Neurology and Rehabilitation Medicine, College of Medicine, University of Illinois at Chicago and 4-Dept. of Neurologyof Neurology, Rush University, Chicago

Multiple Sclerosis (MS) is a demyelinating disease of the central nervous system of unknown etiology. In the relapse remitting form, remyelination and functional recovery follow acute demyelinating episodes. A Growing growing body of literature has shown that there is an increase of neurodegeneration in Relapse Remitting Multiple Sclerosis (RRMS) patients.

Neural stem cells (NSCs) are a subtype of immature, proliferating cells in the nervous system that can self – renew and generate both neurons and glia. In the Adult adult brain, NSCs have been found in the two principal adult neurogenic regions, the hippocampus and the subventricular zone (SVZ) lining the lateral ventricles and in some non-neurogenic regions, including the spinal cord. Recent findings have also shown that peripheral blood, cord blood and bone marrow could generate, under appropriate circumstances, stem cells with neural potential.

In this study we have challenged the idea that cells with neural properties may circulate in the blood of RRMS patients suffering of Relapsing Remitting Multiple Sclerosis (RRMS). Our work recruited 16 patients with RRMS and 23 healthy controls. Peripheral Blood Mononuclear Cells (PBMCs) were isolated through gradients of ficoll- paque and used for RNA and protein analyses. Gene array hybridization, real time PCR and immunoblotting showed up-regulation of the presence of transcripts and protein products encoding for the basic helix-loop-helix transcription factors, Olig1 and Olig2. Experiments are underway to determine the origin and function of these cellsTo rule out the possibility that the expression of these gene might occur in some external sources we tested exosomes from the blood of the patients. mRNA and protein analysis were negative for these genes.

Taken together, our results suggest that PBMCs from RRMS patients contain a subpopulation of cells expressing neuronal genes. The functional role of these cells in disease is discussed.

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# BLOODBORNE CD133/CD34 PRIMITIVE PROGENITOR CELLS INCREASE AFTER RELAPSES IN MULTIPLE SCLEROSIS.

Ariel Finkielsztein[1], Anne I. Boullerne[2], Maria I. Givogri[1], Viviana Ulloa Jofre[1], Agnieszka Kaminska[1], Elizabeth Hartman[3], Demetrios Skias[3], Roumen Balabanov[4], Douglas L. Feinstein[2, 5] and Ernesto R. Bongarzone[1].

[1]Departments of Anatomy and Cell Biology[1], Anesthesiology[2] and Neurology and Rehabilitation Medicine[3], College of Medicine, University of Illinois at Chicago; [4]Multiple Sclerosis Clinic, Rush University; [5]Jesse Brown VA Medical Center.

**CORRESPONDING AUTHOR**: Dr Ernesto R. Bongarzone, Department of Anatomy & Cell Biology, College of Medicine, University of Illinois at Chicago. 808 S. Wood St M/C 512, Chicago, IL, USA. Telephone 312-996-6791. Email ebongarz@uic.edu

AF, AB, MIG and VUJ contributed equally to this work.

***Running Title:*** *CD133+CD34+ stem cells in multiple sclerosis*

***Keywords:*** *multiple sclerosis, neuroinflammation, primitive progenitor cells, stem cells, neurogenesis, hematopoiesis, T-cells, natalizumab, interferon beta.*

## ABSTRACT

Multiple sclerosis (MS) is a demyelinating disorder affecting both men and women, characterized by acute inflammation in the central nervous system (CNS) and followed by remyelination attempts. We hypothesized that circulating stem cells expressing the cell surface marker CD133 are relevant to MS during the CNS repair process. The abundance of CD133+ and CD34+ cells in the peripheral blood of relapsing remitting multiple sclerosis (RRMS) patients and healthy controls was assessed by flow cytometry. CD133+ cells but not CD34+ cells were significantly decreased in RRMS patients ($P$=0.033). Furthermore, levels of CD133+ cells were significantly lower in RRMS women than in RRMS men ($P$=0.014). However, the circulating levels of CD133+CD34+ primitive progenitor cells (pPC) were significantly elevated in RRMS patients ($P$=0.0328). pPC levels correlated with the time elapsed since the last relapse, with higher numbers of pPC following recent relapses. Functionally, RRMS CD133+ cells did not differ from control CD133+ cells, producing the same type and frequency of differentiated hematopoietic colonies, and similar proliferation and migration responses following stimulation with appropriate growth factors. Lower levels of circulating CD133+ cells and increased number of pPC may indicate a shift of this primitive population towards the production of hematogenous lineages involved in immune responses in RRMS.

## INTRODUCTION

Multiple Sclerosis (MS) is a widespread neurological disorder (Rosati, 2001; Compston and Coles, 2008; Trojano et al., 2012; Evans et al., 2013) affecting more women than men (~3 to 1) (Compston and Coles, 2008). To date, MS is without a defined etiology and is considered an autoimmune disorder affecting the central nervous system (CNS), adopting various degrees of severity from relapsing remitting (RRMS) to progressive forms.

Demyelinated lesions or “plaques” are found randomly distributed in the MS CNS (Frohman et al., 2006) and evidence of spontaneous remyelination has been presented (Keirstead and Blakemore, 1999; Barnett and Prineas, 2004; Prineas and Parratt, 2012). However, the cellular and molecular mechanisms regulating remyelination are still largely unclear. Various sources of remyelinating cells may contribute to repair. For example, adult quiescent oligodendrocyte progenitor cells and other neural precursor cells (e.g. derived from the subventricular zone) appear to be recruited for myelin repair (Patrikios et al., 2006; Nait-Oumesmar et al., 2007; Nait-Oumesmar et al., 2008). In addition, infiltration of bloodborne cells in the MS brain underlines the possibility that circulating undifferentiated precursors may also contribute to neural repair (Porat et al., 2006), similarly to bloodborne monocytes colonizing the brain and becoming *bona fide* microglia (Soulet and Rivest, 2008). The blood contains a population of highly proliferative primitive progenitor cells (pPC) characterized by the co-expression of cell surface markers CD34 and CD133 (Krause et al., 1996; Miraglia et al., 1997). CD133, a 120-kDa glycosylated pentaspan integral surface

protein (Miraglia et al., 1997; Mizrak et al., 2008), identifies a population of undifferentiated precursor cells present in various tissues, including blood, fetal liver, kidney, prostate and CNS (Yin et al., 1997; Lee et al., 2005; Corti et al., 2007; Mizrak et al., 2008). Circulating CD133+CD34+ pPC are pluripotential being capable to generate hematopoietic lineages and also neural lineages (Uchida et al., 2000; Schwartz et al., 2003; Jang et al., 2004; Hombach-Klonisch et al., 2008; Kuci et al., 2008; Hafizi et al., 2013). Because of this plasticity, progenitor cells expressing CD133 are of interest for their potential role in neural repair. To what extent CD133+ cells are affected in MS is unclear.

This study measured the *in vivo* abundance and *in vitro* functionality by assessing the proliferative, migratory and differentiation responses of circulating CD133+/CD34+ cells isolated from RRMS patients.

## MATERIALS AND METHODS

**Ethics statement.** The study was approved by the Office for the Protection of Research Subjects of the Institutional Review Board at University of Illinois at Chicago (# 2001-0721), and by the Research and Clinical Trials Administration Office of the Institutional Review Board at Rush University Medical Center (# 09092107-IRB01). Informed written consent was signed by each subject.

**Patients and clinical data.** We prospectively enrolled RRMS patients diagnosed according to the revised McDonald's criteria (McDonald et al., 2001; Polman et al., 2011). Age matched healthy volunteers were selected from students, University personnel, and colleagues of the laboratory and the hospital. These controls were probed to exclude a diagnosis of MS or other neurological diseases. Blood samples from 20 RRMS patients (15 women and 5 men; mean ± standard deviation age of 42 ± 9 years) and 23 healthy volunteers (11 women and 12 men; 38 ± 12 years) were used for flow cytometry. Four patients were in the midst of a relapse at the time of blood draw while others were in remission for 2 months to 5 years. Blood samples from 8 additional RRMS patients (2 women and 6 men; 43 ± 9 years) and from 4 controls (2 women and 2 men; 38 ± 10 years) were used for *in vitro* experiments. These additional patients were all in remission for 1 to 3 years. Table 1 summarizes the demographic and clinical information of all MS patients.

**Isolation of peripheral blood mononuclear cells.** Blood was drawn by venipuncture between 9 am and 2 pm (before lunch) by qualified personnel at the MS clinic of Rush University and the Neurology clinic of University of Illinois at

Chicago. PBMCs were isolated from 15 to 30 ml blood collected in EDTA tubes (BD, Franklin Lakes, NJ) using the Ficoll-Paque protocol. Briefly, blood was diluted 1:1 in sterile 2 mM EDTA PBS to decrease viscosity. A gradient of Ficoll-Paque (GE Healthcare Biosciences, Pittsburgh, PA) was prepared by centrifugation with a swinging bucket centrifuge model 5810 (Eppendorf, Hamburg, Germany) at 1,000 g for 10 min, using an acceleration value of 9, in frit-containing tubes (Greiner Bio-One, Kremsmuenster, Austria). Diluted blood was loaded onto the gradient and spun at 1,000 g for 15 min without applying brake. PBMCs were carefully recovered and washed twice in 10 ml of 2 mM EDTA, PBS by spinning first at 300 g, then at 200 g to eliminate Ficoll-Paque and platelets. Cells were counted in a Neubauer chamber (Reichert, Buffalo, NY) and adjusted to 1 million cells per ml in 0.5% BSA, 2 mM EDTA, PBS.

**Flow cytometry.** PBMCs were pelleted at 3,000 g for 5 min and resuspended in 100 µl of human FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). Each tube containing one million PBMCs was incubated for 15-30 min at 4°C with 1 µg of either a mouse anti-human CD133 antibody-PE (phycoerythrin), a mouse anti-human CD34 antibody-FITC (fluorescein isothiocyanate), or both. Negative controls included incubations with IgG1-PE and IgG2a-FITC. All antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). After two washes at 300 g, cells were transferred to fluocytometry tubes and incubated with 7-Amino-Actinomycin D (BD, Franklin Lakes, NJ) to label dead cells, which were subtracted from the total cell count. About 200,000 events were counted in G2 using a LSR I flow cytometer (Beckton

Dickinson, Franklin Lakes, NJ). Counted cells were expressed as a percentage of the total gated lymphocytic fraction of PBMCs. Raw data analysis was performed with Summit Version 4.3 software (Dako, Glostrup, Denmark). The fraction of double labeled CD133+CD34+ cells from total CD133+ cells was calculated by first subtracting the appropriate controls (IgG-PE and IgG-FITC) from the corresponding fluorescent CD133 and CD34 values. The corrected values were used in the percentage formula: [(CD133+CD34+)/(CD133+)] X 100.

**Magnetic bead sorting.** CD133+ cells were purified from PBMCs using a CD133 MicroBead Kit for human cells and Magnetic-Activated Cell Sorting (MACS®) separation columns (both from Miltenyi Biotec), following the manufacturer's recommendations. Typically, 100,000 to 200,000 cells were isolated per subject for immediate use in the assays described below.

**Colony Forming Unit Assay.** The colony forming unit assay was performed on CD133+ cells purified by MACS from 3 RRMS patients and 3 healthy volunteers, immediately mixed in medium and plated in three independent experiments in triplicate. Cells were cultured in human Methocult® medium H4434 Classic containing the recombinant human growth factors erythropoietin, stem cell factor, interleukin-3 and granulocyte macrophage colony stimulating factor, all from StemCell Technologies (Vancouver, Canada). Briefly, about 10,000 CD133+ cells per patient or control were resuspended in 300 μl of Iscove's Modified Dulbecco's Medium supplemented with 2% fetal bovine serum. Six ml of Methocult medium was slowly added to the cell suspension, which was plated using 16 gauge blunt-end needles in triplicates of 35 mm dishes (3,000 cells per

2 ml). Cultures were incubated at 37°C under 5% $CO_2$, and left undisturbed per the manufacturer instructions. After a 15-day culture, the frequency and type of colonies from each of the 3 independent experiments (3 MS and 3 controls per experiment) performed in triplicate were scored and grouped according to their morphological features.

**Cell Proliferation Assay.** Cell proliferation assay was performed by plating 180,000 MACS isolated CD133+ cells from a subject in a six-well plate coated with a layer of Poly-L-lysine (10 μg/ml) underneath a top layer of laminin (10 μg/ml) at a density of 30,000 cells per well. Cells were first plated in Minimum Essential Medium (MEM), and allowed to attach overnight to the well bottom in a 37°C, 5% $CO_2$ incubator. One half of the plate (3 wells) was then supplemented with growth factors to promote cell proliferation: 150 ng/ml of Fms-like tyrosine kinase-3 ligand (FLT3-Ligand; R&D Systems, Minneapolis, MN), 150 ng/ml of stem cell factor, and 20 ng/ml of interleukin-3 (Cell Signaling, Danvers, MA) in CellGro® serum-free stem cell growth medium (CellGenix, Freiburg, Germany). The other half of the plate (3 wells) was incubated in CellGro® medium and omitting the growth factors as control reference. Cells were cultured for 6 days before being photographed, measured for viability, and counted using the ImageJ software subroutine. Six independent experiments (3 MS patients and 3 healthy subjects) were carried in triplicate.

**Cell Migration Assay.** Cell migration assay was performed using a 96-well format Neuroprobe ChemoTX® Disposable Chemotaxis System with a filter pore size of 8 μm (Receptor Technologies, Adderbury, U.K.). Briefly, 180,000

CD133+ cells in PBS, MACS purified from 3 MS patients and 3 healthy volunteers, were incubated with a 10 μM solution of calcein AM pre-dissolved in DMSO (Molecular Probes, Life Technologies, Grand Island, NY). After 15 min incubation, cells were washed twice in PBS by centrifugation at 300 g at 4°C for 5 min, and resuspended in MEM at a concentration of 30,000 cells per 50 μl. Then 29 μl of plain MEM medium or MEM plus 10 ng/ml stromal cell-derived factor-1α (SDF-1α, R&D Systems) was added to the wells. The 8 μm pore filter was placed on top of the 96-well plate and 50 μl of the calcein-labeled cell suspension was placed above the filter membrane according to the manufacturer's specifications. The 96-well plate andfilter unit was covered with the provided lid and incubated at 37°C for 2h under 5% $CO_2$. After two hours the fluorescent signal of the cells at the bottom of wells were measured at 515 nm in a plate reader. Six independent experiments (3 MS patients and 3 healthy subjects) were carried in triplicate.

**Statistics**. All values are expressed as mean±standard deviation of percentages. All statistical analyses were performed using non-parametric tests: Mann-Whitney U Test, Spearman correlation and Kruskal-Wallis analysis of variance (GraphPad Prism).

## RESULTS

**CD133+ cells are less abundant in the peripheral blood of RRMS patients.** Total levels of CD133+ cells were quantified by flow cytometry. CD133+ cells were found significantly (*P*=0.033) decreased in our group of RRMS patients (0.17% ± 0.08%, N=20), when compared to controls (0.34% ± 0.27%, N=23) (Figure 1A). Interestingly, a gender effect appears to influence the abundance of CD133+ cells in RRMS. In controls, abundance of CD133+ cells in women (0.30% ± 0.20%, N=11) was not significantly different from that in men (0.38% ± 0.32%; N=12, *P*=0.74) (Figure 1B). In contrast, there was a significant (*P*=0.014) decrease in CD133+ cells in RRMS women (0.15% ± 0.07%, N=15) versus RRMS men (0.24% ± 0.05%, N=5) (Figure 1B). Control women had about twice the levels of CD133+ cell than RRMS women (*P*=0.034) while levels of CD133+ cells were not significantly different between control and RRMS men, (Figure 1B). Taken together, these results indicate a decrease in CD133+ cells in RRMS patients, which is more pronounced in affected women. In contrast, total levels of CD34+ cells were not affected in RRMS patients (Figure 1C) and no gender effect was observed (Figure 1D).

**Changes in total CD133+ cells do not correlate with clinical variables and ongoing treatments in RRMS patients.** Spearman analyses were performed to study for possible correlations between levels of peripheral circulating CD133+ cells and age of patients, EDSS, disease duration and time elapsed from last relapse. Table II shows these quantitative data. Although CD133+ cells were

reduced in RRMS patients, this reduction did not correlate directly with any of the clinical parameters. Levels of CD34+ cells correlated with time elapsed from last relapse (*P*=0.0177) but did not correlate with other clinical parameters (Table II).

We examined whether the treatments given to RRMS patients had any effect on the circulating levels of CD133+ or CD34+ cells. Total counts of CD133+ cells were not significantly different in patients treated with interferon-β (Avonex, Betaseron, Rebif), natalizumab (Tysabri), Glatiramer acetate or untreated (*P*=0.15), despite being significantly reduced in comparison to CD133+ cell levels in controls (Figure 1E). CD34+ cells were also non-significantly different in RRMS patients undergoing different treatments (Figure 1F). The stability of circulating levels of total CD133+ cells was assessed in three patients across 2 time points separated by 6 to 9 months (Figure 1G). CD133 levels fluctuated modestly and non-significantly across time. One patient transitioned from interferon-β to natalizumab therapy between the two-time point study and experienced a somehow larger and positive fluctuation of CD133+ cells but this was still not significant.

**Increased levels of CD34+CD133+ pPC in peripheral blood of RRMS patients.** pPC are identified by their co-expression of cell surface markers CD133 and CD34 and are a significant source of rapid proliferating self-renewing pluripotential stem cells in a variety of tissues (Ratajczak et al., 2004). The participation of pPC in various diseases has also been reported, pointing to potential roles in regeneration and repair (Bühring et al., 1999). Little is known

about the status of pPC in the blood of RRMS patients. Analysis of the subpopulation of CD133+ cells co-expressing CD34 showed a significant ($P$=0.0328) increase of pPC in the peripheral blood of RRMS patients within this study (Figure 2A). Furthermore, a significant ($P$ =0.0226) correlation was found between the abundance of pPC in blood and the time elapsed since the last relapse (Figure 2B, Table II). RRMS patients showed higher levels of pPC immediately after or close to a relapse. Interestingly, we found that RRMS patients lost any correlation between the abundance of pPC and their age in contrast to controls (Figure 2C, 2D, Table II), underlining the possibility that relapses stimulate the levels of circulating pPC.

**RRMS and control CD133+ progenitors show similar hematopoietic and proliferative responses *in vitro*.** We investigated for potential *in vitro* functional differences of CD133+ cells from RRMS patients. We first used a semi-solid methyl-cellulose assay and induced CD133+ cells to generate all distinct hematopoietic lineages in the presence of erythropoietin, stem cell factor, interleukin-3 and granulocyte macrophage colony stimulating factor. Although CD133+ cells from RRMS patients were capable of forming all expected colonies, we found no significant differences in colony morphology (Figure 2E-2H) and frequency (Figure 2I) with respect to colonies formed by CD133+ cells isolated from healthy donors. Potential differences in proliferative capacity of CD133+ cells were studied in serum-free medium formulated to stimulate the proliferation of stem cells. Although CD133+ cells from healthy donors responded

significantly (*P*=0.0159) to the mitogenic stimulus, this proliferative response was not significant in RRMS cells (Figure 2J). Finally, we measured the *in vitro* response of CD133+ cells to migratory cues mediated by the SDF1-α-CXCR4 signaling axis. For this, migration of CD133+ cells across a porous transwell filter was measured after stimulation with SDF1-α for 2 hours. We did not find significant differences in the migration response of RRMS CD133+ cells with respect to cells from control donors (Figure 2K).

## DISCUSSION

In this study, we analyzed the circulating levels of bloodborne stem cells identified by their expression of CD133 and CD34 surface markers in a cohort of 20 RRMS patients. Our analyses showed a significant reduction of total levels of CD133+ cells with no significant change in the levels of CD34+ cells. However, the pPC subpopulation of stem cells identified by the co-expression of CD133 and CD34 markers were significantly increased in RRMS patients, particularly in those that underwent a recent relapse. These results suggest that levels of CD133+ cells and particularly those of CD133+CD34+ pPC may help identify RRMS patients who may be close to a relapse or with higher risk of developing a relapse.

The RRMS patients who volunteered for this study had first line and second line medications, including IFN-β, natalizumab, and Glatiramer Acetate. For the last twenty years, most RRMS patients start a treatment immediately following diagnosis. Hence, the availability of untreated RRMS patients was scarce to null at the time of enrollment in our study. Non-treated MS patients exist among the progressive forms of the disease. However, these were not included in our study because progressive forms (primary and secondary) are different than RRMS (Arnason, 2011), which would have introduced biased results on their CD133+ cells status when comparing with RRMS patients.

CD133+ stem cells circulate in minute numbers but are pluripotent. Active research aims to study their function in an array of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, especially for their

angiogenesis regenerative properties. In MS, CD133+ stem cells are of interest for both hematopoiesis and neurogenesis. CD133 is a pentaspan protein expressed in stem cells from a variety of tissues, including hematopoietic (Miraglia et al., 1997; Yin et al., 1997) and neural cells (Porat et al., 2006). As such, CD133+ have been isolated from the mouse and human brain and showed their ability to generate neurons, oligodendrocytes and astrocytes. Because CD133 expression marks also a circulating population of pluripotential stem cells in the blood, the possibility that bloodborne CD133+ stem cells may participate in demyelination-remyelination in MS cannot be excluded. The characterization of CD133+ populations in this study is a first step towards understanding the potential role of these cells in MS.

Our results showed a significant reduction of the total levels of CD133+ cells in the blood of RRMS patients. The reduction of CD133 levels may represent a reduced proliferation of these cells. For example, IFN-β affects the *in vitro* proliferation of peripheral mononuclear cells (Noronha et al., 1993). Patients under IFN-β treatment have shown decreased numbers of newly produced T lymphocytes and the association between reduced thymic output and low peripheral T lymphocytes can be a cause of leukopenia, a frequent side effect of the IFN-β therapy (Zanotti et al., 2011). Because about half of the patients in this study were under IFN-β treatment, this possibility cannot be completely excluded. However, our comparison of the CD133+ levels between IFN-β-treated patients, natalizumab-treated patients, and a heterogeneous group treated with Glatiramer Acetate or no treatment showed no significant differences among the various

groups of RRMS patients. This suggests that the decrease in blood levels of CD133+ cells is likely a feature of these patients. Furthermore, the absence of significant changes in CD34+ cells also points to the absence of a generalized repression by IFN-β. However, other factors may have contributed to lower the levels of bloodborne CD133+ cells in RRMS patients, namely (i) CD133+ cells may undergo higher rates of cell death in RRMS; (ii) CD133+ cells may have lower or slower proliferation rates, leading to low amplification of the circulating pool; (iii) CD133+ cells may migrate from blood into solid tissues such as the CNS and (iv) CD133+ cells may differentiate more rapidly into hematopoietic and/or other cell lineages. It is tempting to speculate that CD133+ cells may be recruited to repair demyelination; however it is not yet known whether CD133+ cells can directly engage in myelin repair in MS. One working hypothesis is that MS patients undergo an active phase of remyelination, during which bloodborne stem cells could contribute directly or indirectly to the repair mechanism. Several reports support the potential of CD133 cells to form neural cell types. For example, human fetal CD133+ neural stem cells and human CD133+ stem cells mobilized from peripheral blood promoted faster recovery after transplantation in a rodent model of spinal cord injury (Sasaki et al., 2009). Similarly, CD133+ cells improve peripheral nerve regeneration (Kijima et al., 2009). Amyotrophic lateral sclerosis patients grafted with autologous CD133+ cells in the frontal motor cortex showed some improvement in quality of life and life expectancy (Martinez et al., 2009). Our results provide initial evidence that at least the hematopoietic potential of RRMS CD133+ cells was not significantly compromised in affected

patients, which would suggest that their neural potential is also intact. Overall, these reports underline the potential role for CD133+ cells in neural repair. Whether CD133+ cells from peripheral blood could be used for improving repair in RRMS brains and whether CD133+ cell-mediated neural repair occurs *in vivo* in the MS brain need further studies.

Our study found that the reduction of CD133+ cells was more pronounced in RRMS women than men. Lower ratios of circulating cells co-expressing CD133 and CD34 have been reported in healthy women than in men (Reichelt et al., 2009). Reduced levels of CD133+ cells in RRMS women may be related to the higher incidence of RRMS in women (3:1) (Compston and Coles, 2008). For example, women affected by the autoimmune disease systemic lupus erythematosus are also characterized by a decrease in circulating CD133+ cells when compared to their healthy counterparts (Moonen et al., 2007). In addition, a larger pool of CD133+ cells in men may be related to sex hormones. Interaction between progesterone and estrogen and stem cells has been described (Brannvall et al., 2002; Hong et al., 2004). Little is known about the status of CD133+ cells in RRMS patients. Hence, to our knowledge, our study is the first to report that circulating CD133+ cells may be a relevant variable correlating gender effects in MS. As such, a larger study of the circulating levels of CD133+ cells in MS patients should be explored to determine its use as a screening tool for patients at risk of developing MS.

Correlation studies between abundance of circulating CD133+CD34+ pPC and age are somewhat controversial (Heiss et al., 2005; Shaffer et al., 2006;

Reichelt et al., 2009). Aging was shown to decrease circulating CD34+ cells, but not those of circulating CD133+ cells in subjects around 60 year-old (Povsic et al., 2010). In our study, we found a significant correlation of pPC levels with age in the control group. Interestingly, this correlation was lost in patients with RRMS, likely indicating the immune response of these patients in response to relapses. It remains to be seen whether progressive MS patients have the same CD133 variability.

The regeneration potential intrinsic for CD133+ stem cells and for pPC in particular is of high relevance. For example, CD133+ cells have the ability to generate both hematopoietic and endothelial progenitors (Gehling et al., 2000). These cells, which also express the CXCR4 receptor, respond to SDF-1$\alpha$ and VEGF and are thought to participate in angiogenesis (Pozzobon et al., 2010; Adini et al., 2013). CD133+ stem cells and pPC are also responsive in a variety of pathological conditions including cancers, vascular and cardiac damage, liver cirrhosis and Duchenne dystrophy (Turan et al., 2007; Marchesi et al., 2008; Gehling et al., 2010; Pescini et al., 2010). Our study found a positive correlation between the abundance of circulating pPC in RRMS patients and relapse, with higher levels of pPC during recent clinical episodes. Levels of pPC in our group of healthy donors is similar to previous reports (Moonen et al., 2007). An increase in circulating levels of pPC in patients with recent relapses may be relevant to promote peripheral and central tissue repair. Alternatively, pPC may be mobilized to replenish the pool of hematopoietic cells infiltrating the CNS

during a relapse. Their involvement in these processes requires further investigation.

In summary, the results presented here demonstrate differences in the circulating population of CD133+ cells in RRMS patients. Our findings of levels of CD133+ cells lower in RRMS women than in RRMS men may be of relevance to understand gender differences in RRMS patients. Increases in pPC levels in relapsing RRMS patients may be another indicator of disease status and recovery.

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## FIGURE LEGENDS

**Figure 1. Decreased levels of circulating CD133+ cells in RRMS patients.** Peripheral blood mononuclear cells from relapsing remitting multiple sclerosis (RRMS) patients and healthy volunteers were analyzed for the abundance of CD133+ and CD34+ cells by flow cytometry. **A)** Comparison between 23 healthy volunteers (Control), and 20 patients (RRMS) showed a significant decrease in CD133+ cells in RRMS patients (*P*=0.033). **B)** Comparison between genders (11 control women, 12 control men, 15 RRMS women and 5 RRMS men) showed significant decreases between RRMS women and men (*P*=0.014) and between control and RRMS women (*P*=0.034). **C)** Comparison between 23 healthy volunteers (Control), and 20 patients (RRMS) did not show significant differences in CD34+ cells. **D)** Comparison between genders (11 control women, 12 control men, 15 RRMS women and 5 RRMS men) also showed no difference in total levels of CD34+ cells. Values are expressed as the mean percentage ± standard error of CD34+ cells in the lymphocyte fraction. **E, F)** The percentage of CD133+ cells (E) and CD34+ cells (F) in the lymphocyte fraction from RRMS patients treated with interferon-β (IFN-β, N=11); natalizumab (Tysabri, N=4); glatiramer acetate, N=3; or no treatment, N=2 (Others) showed no significant difference among treatments on levels of either cell population (Kruskal-Wallis, *P*=0.15). **G)** The stability of circulating levels of CD133+ cells was assessed in three RRMS patients across 2 time points several months apart. MS5 patient (man) transitioned from interferon-β to natalizumab while MS6 (woman) and MS7

(man) patients remained on interferon-β. Bars represent percentages of CD133+ cells in the lymphocyte fraction.

**Figure 2. CD133+CD34+ pPC are increased in RRMS patients and correlate with relapses. A)** Peripheral blood mononuclear cells from relapsing remitting multiple sclerosis (RRMS) patients and healthy volunteers were analyzed for the abundance of double positive CD133/CD34 primitive progenitor cells (pPC) by flow cytometry. The percentage of CD133+CD34+ pPC was analyzed in 17 healthy controls and 13 RRMS patients. RRMS patients showed significantly higher numbers of double-stained CD133+CD34+ pPC (69.9% ± 27.4%) than controls (47.8% ± 22.2%, Mann-Whitney, *P*=0.0328). **B-D)** A correlation was found between the abundance of pPC in RRMS patients and time elapsed since the last relapse (B, *P*=0.0226) but not with the age of the patient (C). Instead, pPC levels correlated with age in control volunteers (D) (*P*=0.0274). **E-H)** Representative images of colonies after culturing CD133+ cells isolated from 3 relapsing remitting multiple sclerosis (RRMS) patients (E,G) and 3 control (F,H) individuals in Methocult medium for 15 days. All photographs were taken at 100X magnification. **I)** Colonies were counted directly from each culture, and the results grouped according to their morphological features. CFU: colony forming unit; CFU-E: erythrocyte; BFU-E: burst-forming unit erythrocyte; CFU-GM: granulocyte monocyte; CFU-GEMM: granulocyte erythrocyte monocyte megakaryocyte; CFU-M: monocyte; CFU-G: granulocyte. Colony frequency is expressed as a percentage of each type of colony of the total of colonies formed

± standard error. No significant differences were determined. **J)** The total number of cells responding to growth factors (GF) was counted six hour after plating CD133+ cells ($3x10^4$ cells per well) in serum-free medium with or without the addition of GF. Significant proliferation (*P*=0.0159) was measured in control CD133+ cells but not in RRMS cells. **K)** Transwell migration was measured two hours after plating CD133+ cells ($3x10^4$ cells per well) pre-labeled with Calcein AM and incubated with or without the addition of stromal cell-derived factor-1$\alpha$ (SDF-1$\alpha$). No significant migration differences between RRMS and control cells were measured. All experiments were performed in 3 independent experiments (3 RRMS and 3 controls) in triplicates.

**Table I. Demographics of 20 RRMS patients for flow cytometry**

| Patient Code | Gender | Age years | EDSS | Treatment Chemical names | Duration years |
|---|---|---|---|---|---|
| MS03 | Female | 47 | 0.5 | Interferon Beta | 10 |
| MS04 | Female | 46 | 6 | Interferon Beta | 7 |
| MS05 | Male | 50 | 7 | Interferon Beta | 14 |
| MS06 | Female | 50 | 6.5 | Interferon Beta | 6 |
| MS07 | Male | 54 | 0 | Interferon Beta | 5 |
| MS08 | Female | 39 | 5.5 | Glatiramer Acetate | 11 |
| MS09 | Male | 43 | 0.5 | Glatiramer Acetate | 6 |
| MS10 | Male | 53 | 5 | Interferon Beta | 5 |
| MS11 | Female | 33 | 0 | Natalizumab | 3 |
| MS12 | Female | 46 | 6 | Interferon Beta | 15 |
| MS13 | Female | 33 | 1 | Interferon Beta | 9 |
| MS14 | Female | 28 | 3 | Interferon Beta | 1 |
| MS15 | Female | 33 | 2 | None | 0.25 |
| MS16 | Female | 27 | 4.5 | Natalizumab | 7 |
| MS18 | Female | 55 | 1 | Glatiramer Acetate | 0.33 |
| MS19 | Female | 30 | 4.5 | Interferon Beta | 2 |
| MS20 | Female | 54 | 5 | Natalizumab | 15 |
| MS21 | Female | 38 | 0 | None | 1 |
| MS22 | Male | 46 | 6.5 | Interferon Beta | 25 |
| MS23 | Female | 38 | 1 | Natalizumab | 7 |
| MS24 | Female | 48 | 1 | Natalizumab | 2 |
| MS25 | Male | 34 | 4.5 | Natalizumab | 8 |
| MS26 | Male | 48 | 2 | Natalizumab | 22 |
| MS26 | Male | 48 | 2 | Natalizumab | 22 |
| MS28 | Male | 53 | 3 | Natalizumab | 5 |
| MS29 | Female | 51 | 0 | Natalizumab | 3 |
| MS30 | Male | 30 | 1 | Natalizumab | 8 |
| MS31 | Male | 34 | 4.5 | Natalizumab | 4 |
| MS32 | Male | 47 | 0.5 | Natalizumab | 14 |

## Table II. Correlation with Clinical Parameters

**AGE OF RRMS GROUP**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| *#XY pairs* | 20 | 20 | 13 | 13 |
| *Spearman "r"* | 0.2833 | 0.3573 | 0.1842 | 0.05817 |
| *p value (two-tailed)* | 0.2262 | 0.122 | 0.5469 | 0.8503 |

**EDSS**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| *#XY pairs* | 20 | 20 | 13 | 13 |
| *Spearman "r"* | -0.06624 | -0.01134 | 0.24 | 0.1972 |
| *p value (two-tailed)* | 0.7814 | 0.9622 | 0.4297 | 0.5183 |

**DISEASE DURATION (DD)**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| *#XY pairs* | 20 | 20 | 13 | 13 |
| *Spearman "r"* | -0.0839 | 0.1279 | 0.189 | -0.1848 |
| *p value (two-tailed)* | 0.7251 | 0.5909 | 0.5364 | 0.5455 |

**EDSS/DD**

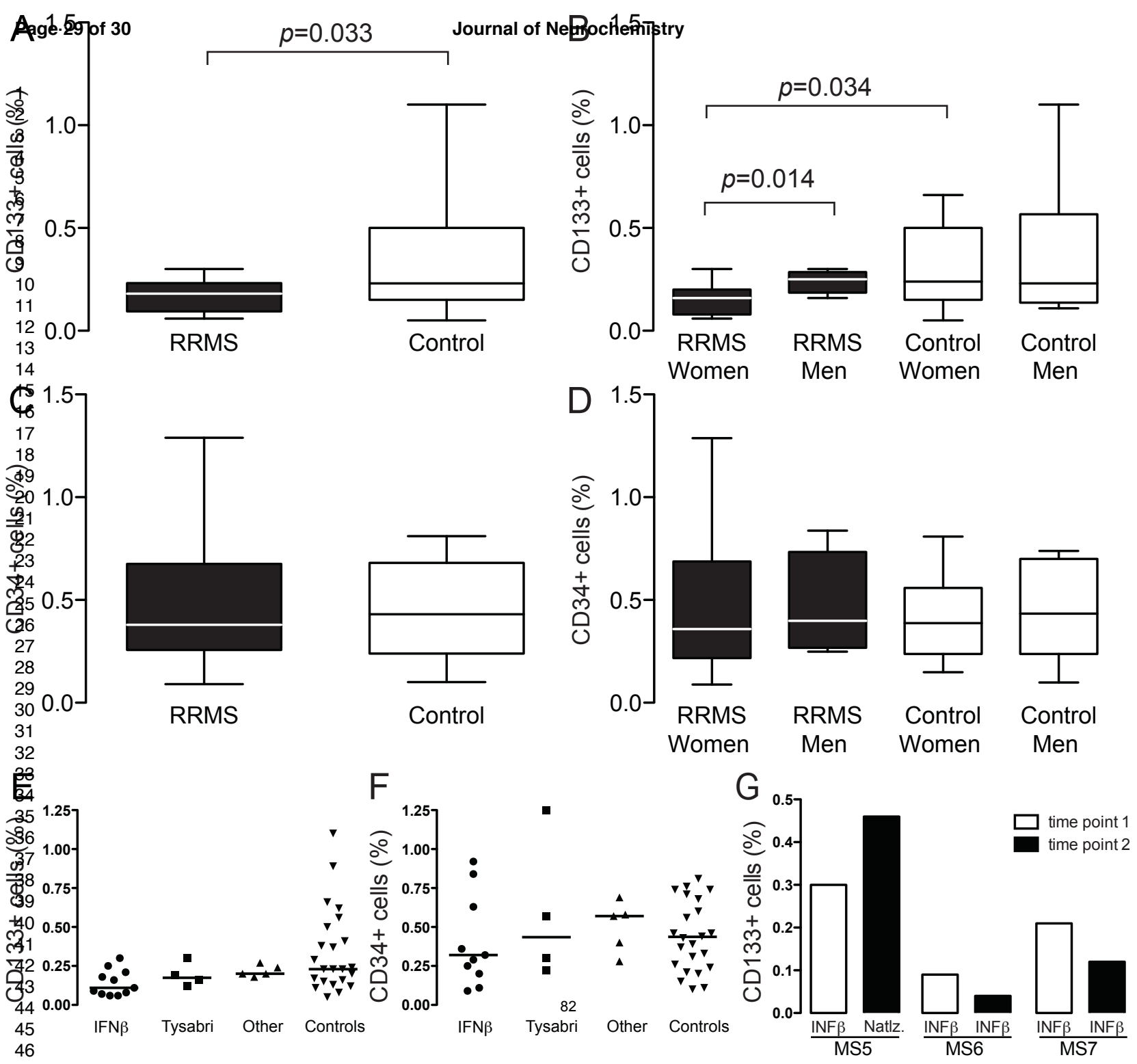
| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| *#XY pairs* | 20 | 20 | 13 | 13 |
| *Spearman "r"* | -0.09585 | -0.03768 | -0.05241 | 0.4221 |
| *p value (two-tailed)* | 0.6877 | 0.8747 | 0.865 | 0.1508 |

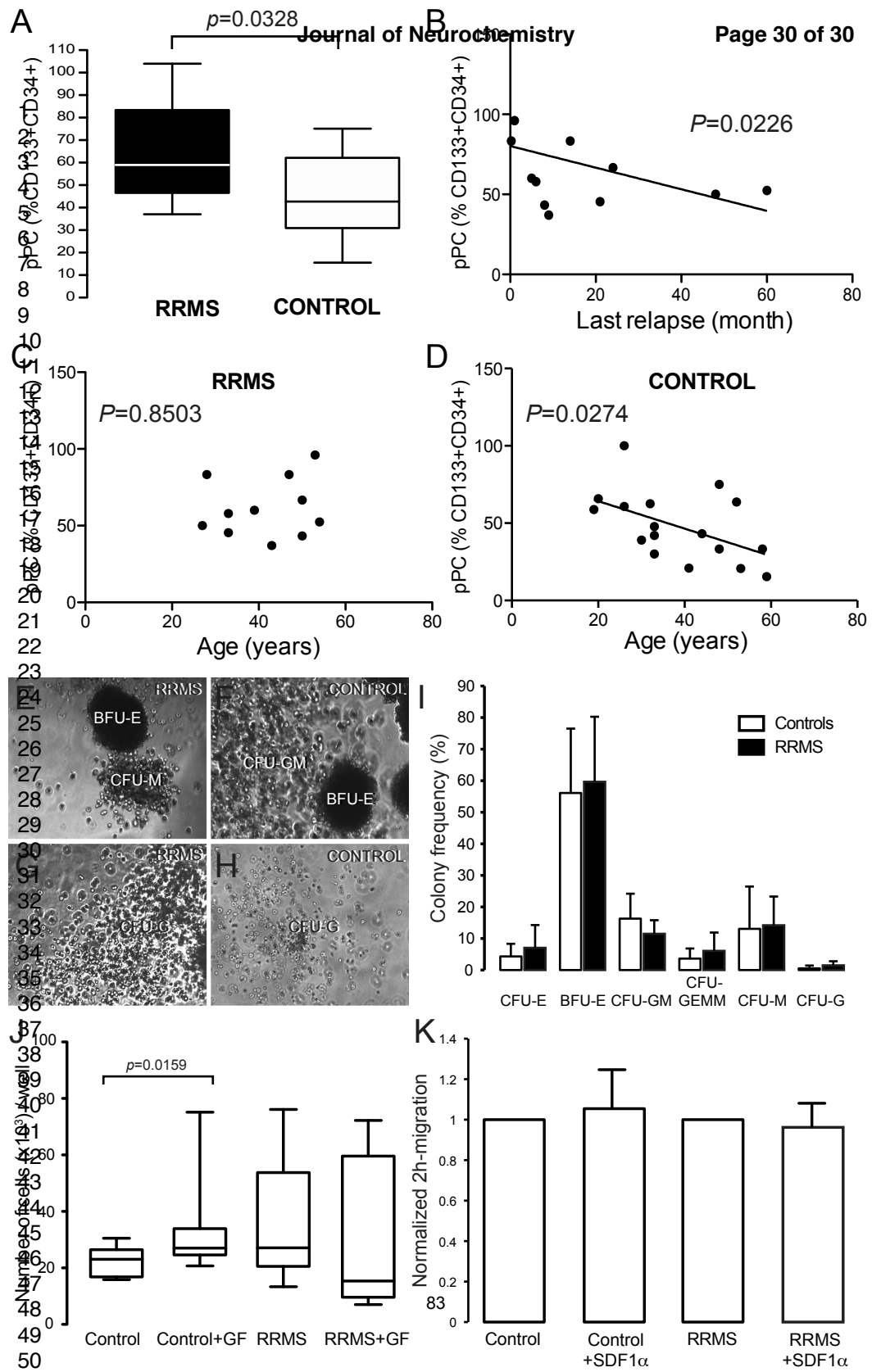
**TIME FROM LAST RELASPSE**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| *#XY pairs* | 20 | 20 | 13 | 13 |
| *Spearman "r"* | -0.0302 | -0.5239 | 0.707 | -0.6107 |
| *p value (two-tailed)* | 0.8994 | **0.0177** | **0.0069** | **0.0226** |

**AGE OF CONTROL GROUP**

| | CD133+ | CD34+ | CD133+/CD34+ | (CD133+CD34+)/CD133+ |
|---|---|---|---|---|
| *#XY pairs* | 20 | 20 | 13 | 17 |
| *Spearman "r"* | -0.1213 | -0.2624 | 0.6363 | -0.5335 |
| *p value (two-tailed)* | 0.5813 | 0.2265 | **0.006** | **0.0274** |


A
p=0.033
CD133+ cells (%)
RRMS
Control
B
p=0.034
p=0.014
CD133+ cells (%)
RRMS Women
RRMS Men
Control Women
Control Men
C
CD34+ cells (%)
RRMS
Control
D
CD34+ cells (%)
RRMS Women
RRMS Men
Control Women
Control Men
E
CD133+ cells (%)
IFNβ
Tysabri
Other
Controls
F
CD34+ cells (%)
IFNβ
Tysabri
Other
Controls
G
CD133+ cells (%)
time point 1
time point 2
INFβ
Natlz.
INFβ
INFβ
INFβ
INFβ
MS5
MS6
MS7


A
p=0.0328
pPC (% CD133+CD34+)
RRMS
CONTROL
B
P=0.0226
pPC (% CD133+CD34+)
Last relapse (month)
C
RRMS
P=0.8503
pPC (% CD133+CD34+)
Age (years)
D
CONTROL
P=0.0274
pPC (% CD133+CD34+)
Age (years)
E
RRMS
BFU-E
CFU-M
F
CONTROL
CFU-GM
BFU-E
G
RRMS
CFU-G
H
CONTROL
CFU-G
I
Controls
RRMS
Colony frequency (%)
CFU-E
BFU-E
CFU-GM
CFU-GEMM
CFU-M
CFU-G
J
p=0.0159
Number of cells (x10³) / well
Control
Control+GF
RRMS
RRMS+GF
K
Normalized 2h-migration
Control
Control +SDF1α
RRMS
RRMS +SDF1α